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Alterations in key metabolic sensors involved in bovine cystic ovarian disease

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ABSTRACT

High-producing dairy cows frequently suffer metabolic alterations that cause different diseases, which could decrease the reproductive efficiency of the herd. Among these reproductive disorders, cystic ovarian disease (COD) has been related to alterations in metabolites and hormonal factors such as insulin, adiponectin and leptin. The aim of this study was to determine the protein expression of adiponectin and some of its downstream targets in ovarian follicles of control cows and cows with clinical diagnosis of COD. We also analyzed some key metabolic sensors in plasma and follicular fluid from both groups. In follicular cysts, we detected higher protein expression of adiponectin receptor 2 (AdipoR2), 5′ adenosine monophosphate-activated protein kinase (AMPK), carnitine palmitoyl transferase 1 (CPT1) and acyl-coenzyme A oxidase 1 (ACOX1) relative to control antral follicles ($p < 0.05$). This was related to higher plasma adiponectin concentration in cows with COD than in control cows ($p < 0.05$). On the other hand, insulin concentrations showed an opposite pattern ($p < 0.05$). Furthermore, we found alterations in local and systemic concentrations of several metabolites. In this regard, in follicular fluid of cystic cows, the concentrations of non-esterified fatty acids and beta-hydroxybutyrate were higher ($p < 0.05$), whereas the concentrations of glucose and triacylglycerol were lower than in follicular fluid from control cows ($p < 0.05$). Besides, in both follicular fluid and plasma of cows with COD, the concentration of cholesterol was higher than in control animals ($p < 0.05$). These results evidence a local altered scenario of some metabolic sensors in cystic follicles, which could generate an adverse microenvironment for the resumption of ovarian activity, possibly causing the persistence of follicles and the recurrence of COD.

1. Introduction

Current production systems involve high-producing dairy cows that face great metabolic demands and environmental stresses that finally compromise their reproductive efficiency [1–3]. One of the most important dysfunctions associated with high-yielding dairy cows is cystic ovarian disease (COD), a disease that compromises normal ovarian cyclicity [4]. In addition to imbalances in different hormones such as insulin, adiponectin, and leptin, several metabolites such as glucose, non-esterified fatty acids (NEFA) and amino acids have been proposed to participate in cyst formation [5]. Therefore, the nutritional and metabolic state of the animal could affect the ovulation process [6–8]. During early lactation, food intake is generally insufficient to meet the demand for nutrients, especially glucose, required to support the extremely high levels of milk production [9]. A nutritional imbalance leads the cow to a state of negative energy balance, in which NEFA from lipid stores of adipose tissue are mobilized and then captured by the liver and oxidized to produce ketone bodies. Beta-hydroxybutyrate (BHB) is the predominant ketone body in blood and its concentration is an index of increased fatty acid oxidation, so it is also considered as a marker of an excessive negative energy balance [10]. Several studies have shown that high levels of NEFA may adversely affect follicular growth and development, resulting cytotoxic and potentially leading to COD [11,12]. This cytotoxicity can lead to an

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alteration throughout folliculogenesis, which prevents recovery from of the disease and favors recidivism in COD presentation. In addition, fatty acids are mainly metabolized by beta-oxidation, a process that includes a series of metabolic reactions with highly regulated enzymes such as carnitine palmitoyltransferase-1 (CPT1) in mitochondria and acyl-coenzyme A oxidase 1 (ACOX1) in peroxisomes [13,14].

Besides being an energy storing tissue, adipose tissue acts as an active endocrine organ that secretes a number of biologically active adipokines involved in multiple physiological processes such as inflammation and lipid and glucose metabolism, with direct and indirect effects on reproduction [15,16]. Adiponectin (known as AdipoQ, Acrp30, apM1, and GBP28) is an adipokine that plays an important role in the control of lipid metabolism, glucose homeostasis and insulin sensitivity, after binding to its two receptors (AdipoR1 and AdipoR2) [14,17,18]. Both adiponectin and its receptors have been identified in bovine granulosa cells, theca cells, corpora lutea, oocytes, and cumulus cells [17]. Once activated, AdipoR1 is more prominent in AMP-activated protein kinase (AMPK) phosphorylation, whereas AdipoR2 is involved in the activation of peroxisome proliferator-activated receptors (PPARs) [19]. AMPK is a metabolic sensor of the cellular energy state and plays an important role in the regulation of lipid, carbohydrate and protein metabolism of peripheral and central tissues [5,19]. Besides, in the ovary, AMPK modulates cellular proliferation and survival, as well as some reproductive functions such as ovarian steroidogenesis and oocyte maturation [14]. Therefore, adiponectin and AMPK may be important fuel sensors required for the growth of follicles, oocytes, and embryos. Based on the above, the aim of this study was to determine the expression of adiponectin and some of its downstream effectors (AdipoR1, AdipoR2, AMPK, CPT1 and ACOX1) in ovarian follicles of control cows and cows with spontaneous COD. We also aimed to evaluate some metabolic sensors such as insulin, glucose, NEFA, triacylglycerol, cholesterol, BHB and adiponectin in plasma and follicular fluid of control cows and cows with spontaneous COD.

2. Materials and methods

2.1. Ethical aspects

All the procedures were evaluated and approved by the Institutional Ethics and Security Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina, protocol N° 134/12, and are consistent with the "Guide for the care and use of agricultural animals in research and teaching, third edition" (Federation of Animal Science Societies, 2010).

2.2. Animals and groups

Multiparous Holstein cows (2–4 parities) from dairy herds of the milk-producing region of Santa Fe, Argentina, were selected for the study. Animals had an average milk production of between 22 and 26 L per day and a body condition score that was between 2.75 and 3.50, and had been in milk for an average of 173–255 days.

Cows were fed a diet based on alfalfa pasture supplemented with corn silage, corn grain, soybean expeller, and hay, following the recommendations of the Nutrient Requirements of Dairy Cattle (2001). Animals were clinically evaluated both before and during the experiments and showed no evidence of diseases other than COD. For this study, we used cows with clinical diagnosis of COD (determined by irregular estrous cycles or anestrus, infructuous inseminations, and the presence of a follicle in one or both ovaries with $\geq$20 mm in diameter, in the absence of a functional corpus luteum in either the right or left ovary and lack of uterine tonicity) [20], and normal cycling cows, synchronized by the modified G6G protocol [21]. The synchronization protocol consisted of two doses of PGF2a (150-mg D-Cloprostenol, Enzaprost D-C; Biogénesis-Bagó, Argentina) administered 12 h apart on Day 0 to induce luteolysis, followed by a dose of GnRH (20-mg buserelin acetate, Gonaxal; Biogénesis-Bagó) 2 days later to stimulate ovulation of the preovulatory follicles present. Six days after the first GnRH dose, the cows started Ovsynch with an injection of GnRH. Seven days later, cows received two doses of PGF2a, 12 h apart, to ensure luteolysis.

Moreover, hormonal steroid determinations were previously performed to correctly classify the follicular cysts, and has been published in previous articles [22,23].

2.3. Ovarian ultrasonography

Ovarian ultrasonographic examinations were performed in all animals by using a real-time B-mode scanner equipped with a 5.0 MHz linear-array transrectal transducer (Honda HS101V, Japan), either to diagnose and corroborate the disease in cows with COD or to monitor synchronization and follicular development in control cows.

2.4. Ovariectomy and follicular fluid aspiration

Bilateral ovariectomy was performed in both groups: after confirmation of diagnosis in cows with COD (n = 10) and at the time of proestrus in control cows (n = 10). Previously, to prevent rupture of cysts, the follicular fluid was aspirated using a digital ultrasound system equipped with a microconvex transducer of 5.0 MHz (Chison 8300vet; Chison Medical Imaging Co., Mainland, China) mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Limited, Sao Paulo, Brazil). Ovariectomy was performed with the animals standing. Briefly, animals were first sedated (Xilazine 2%, Over®) and then anesthetized with infiltrative local anesthesia (Lidocaine 2%, Over®), according to the modified Magda technique [24], and additionally anesthetized with low epidural anesthesia (Lidocaine 2%, Over®). After preparing the surgical area, a vertical incision in the left paralumbar fossa was made covering the skin, and subcutaneous and muscular tissue. Once inside the abdominal cavity, a hemostatic forceps was placed in the ovarian pedicle, then the ovarian vessels were ligated and finally the pedicle was sectioned for the extraction of the ovaries. Finally, the muscle planes, subcutaneous tissue and skin were sutured with nylon thread and the animals were treated with antibiotics, coagulants, anti-inflammatories and external antiparasitics.

In addition, the follicular fluid of other 29 cows with spontaneous COD and 20 control cows in proestrus, previously synchronized by the G6G protocol, was aspirated for metabolite and hormone determinations.

2.5. Sample processing

For histological examination and immunohistochemistry (IHC), ovaries obtained by ovariectomy were sectioned and fixed in 4% buffered formaldehyde for 8–10 h at room temperature after obtaining samples of the complete wall of antral follicles or follicular cysts for molecular biology analysis. Then, the fixed tissues were washed, dehydrated and embedded in paraffin wax. Sections (4 μm thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis, MO, USA) and assigned for staining with hematoxylin and eosin for preliminary observations of all ovarian structures [22] or for use in IHC. Follicles were classified by the criteria established by Braw-Tal and Yossefi [25] into the following categories: primary, small preantral, large preantral, antral and atretic follicles. Follicular cysts were

characterized by a relatively thin wall with the presence of a granulosa cell layer and a vascularized theca interna without signs of luteinization [26,27].

For metabolite assays, follicular fluid samples were collected, cooled on ice and immediately transported to the laboratory and centrifuged to separate the pellet of cells from the follicular fluid. The follicular fluid obtained was stored at −80 °C until analysis.

For metabolite determination, blood samples were collected from both groups on the day of follicular aspiration, via coccygeal venipuncture in tubes containing EDTA and centrifuged. The plasma obtained was stored at −20 °C until analysis.

2.6. Determination of NEFA, BHB, glucose, triacylglycerol and cholesterol concentrations

Plasma and follicular fluid concentration of NEFA was assessed by spectrophotometry by using a commercial kit (Randox Laboratories Ltd., UK), whereas the concentrations of glucose, triacylglycerol and cholesterol were measured with commercial kits (Wiener Lab., Argentina). The reactions were read on a microplate reader (SPECTROstar Nano, BGM LABTECH, Ortemberg, Germany). The concentration of BHB was assessed in whole blood and follicular fluid by using reactive strips (FreeStyle Optium Xceed, Abbott Diabetes Care Ltd., Oxon, UK).

2.7. Determination of insulin concentration

Plasma and follicular fluid concentration of insulin was analyzed by radioimmunoassay, as previously described [28]. Briefly, insulin concentration was measured using anti-bovine insulin antibody (Sigma, St. Louis, MO, USA) and standard human insulin (Laboratorios Beta, Buenos Aires, Argentina); the minimum detectable concentration was 0.05 ng/mL. The intra-assay and inter-assay coefficients of variation were always lower than 8% and 11%, respectively.

2.8. Determination of adiponectin concentration

Plasma and follicular fluid concentration of adiponectin in all cows was determined using a commercial ELISA kit (Bovine Adiponectin ELISA Kit, LifeSpan BioSciences, Inc., Seattle, USA; detection limit of 0.781 ng/mL), following the manufacturer's instructions. All plasma and follicular fluid samples were prediluted at a concentration of 1:100 with phosphate buffered saline (PBS) 0.01 M pH 7–7.2, and then brought to a final concentration of 1:1000 by using the kit sample diluent. The colorimetric reactions were read on a microplate reader (SPECTROstar Nano).

2.9. Immunohistochemistry (IHC)

Histological ovarian sections from all cows were used for IHC to locate and quantify the protein expression of AdipoR1, AdipoR2, AMPK, CPT1 and ACOX1 in the follicular wall. After deparaffinization and hydration, endogen peroxidase activity was inhibited with 3% (vol/vol) H_2O_2 in methanol, and nonspecific binding was blocked with 10% (vol/vol) normal goat serum (AMPK, CPT1 and ACOX1) or normal donkey serum (AdipoR1, AdipoR2). All sections were incubated with primary antibodies. The conditions of the IHC and suppliers of the primary and secondary antibodies used are reported in Table 1. The antigens were visualized by the CytoScan™ HRP Detection System (Cell Marque, Rocklin, CA, USA), and 3,3-diaminobenzidine (Liquid DAB-Plus Substrate Kit, Zymed, San Francisco, CA, USA) was used as chromogen. Finally, the slides were washed in distilled water and counterstained with activated hematoxylin (Biopur, Rosario, Argentina), dehydrated and mounted. To verify the immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing the primary antibody with goat or donkey non-immune sera. The specificity of the secondary antibodies was tested by incubation with anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako, Carpinteria, CA, USA), a primary antibody against human antigens with a proven negative reaction to cattle tissues. To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB reagent alone [29]. Positive controls were used as inter-assay controls to maximize the levels of accuracy and robustness of the method [30,31].

2.10. Western blotting

The specificity of the primary antibodies against AdipoR1, AdipoR2, AMPK, CPT1 and ACOX1 used in IHC was evaluated by western blot, using specific primary antibodies. Bound antibodies were detected using goat anti-rabbit IgG HRP antibody (Santa Cruz, sc-2004) or donkey anti-goat IgG HRP antibody (Santa Cruz, sc-2020) (Table 2). The immunopositive bands were detected by chemiluminescence using the ECL-plus system (GE Healthcare) on

Table 1
Antibodies, suppliers and conditions used for immunohistochemistry (IHC) analysis.

Primary Antibodies		Antigen Retrieval	Blockade	Secondary Antibodies (1/100)	
Type and supplier	Conditions			Type and supplier	Conditions
AdipoR1 Polyclonal (C-14:sc-46748, Santa Cruz Biotechnology, Inc., CA, USA)	1/100 O.N. at 4 °C	Conventional microwave pretreatment	10% (vol/vol) normal donkey serum	Donkey-antigoat IgG (Santa Cruz Biotechnology, Dallas, Texas, USA)	1/100 30 min. at 25 °C
AdipoR2 Polyclonal (M-16: sc-46754, Santa Cruz Biotechnology, Inc., CA, USA)	1/50 O.N. at 4 °C	Intense microwave pretreatment	10% (vol/vol) normal donkey serum	Donkey-antigoat IgG (Santa Cruz Biotechnology, Dallas, Texas, USA)	1/100 30 min. at 25 °C
AMPKα1/2 Polyclonal (H-300:sc-25792, Santa Cruz Biotechnology, Inc., CA, USA)	1/500 O.N. at 4 °C	Pressure cooker pretreatment	10% (vol/vol) normal goat serum	Goat-antirabbit IgG (Santa Cruz Biotechnology, Dallas, Texas, USA)	1/100 30 min. at 25 °C
CPT1-L Polyclonal (H-95:sc-20669, Santa Cruz Biotechnology, Inc., CA, USA)	1/50 O.N. at 4 °C	No antigen retrieval	10% (vol/vol) normal goat serum	CytoScan biotinilated link, CytoScanTM HRP detection system (Cell Marque, CA,USA)	Ready to use 30 min. at 25 °C
ACOX1 Polyclonal (H-140: sc-98499, Santa Cruz Biotechnology, Inc., CA, USA)	1/800 O.N. at 4 °C	Conventional microwave pretreatment	10% (vol/vol) normal goat serum	CytoScan biotinilated link, CytoScan TM HRP detection system (Cell Marque, CA,USA)	Ready to use 30 min. at 25 °C

O.N.: overnight. min: minutes.

Table 2
Antibodies, suppliers and conditions used for western blotting analysis.

Primary Antibodies		Secondary Antibodies	
Type	Conditions	Type and supplier	Conditions
AdipoR1	1/500	Donkey anti-goat IgG HRP Santa Cruz (sc-2020)	1/40,000
AdipoR2	1/100	Donkey anti-goat IgG HRP Santa Cruz (sc-2020)	1/40,000
AMPKα1/2	1/500	Goat anti-rabbit IgG HRP Santa Cruz (sc-2004)	1/7500
CPT1-L ACOX1	1/250	Goat anti-rabbit IgG HRP Santa Cruz (sc-2004)	1/10,000
	1/500	Goat anti-rabbit IgG HRP Santa Cruz (sc-2004)	1/10,000

hyperfilm-ECL film (GE Healthcare) [30].

2.11. Image analysis

Microscopic images were digitized using a color video camera Nikon DS-Fi2 mounted on a conventional light microscope Nikon Eclipse Ci-L *Ni* (Tokyo, Japan), with an objective magnification of ×40, and then analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA), as described and validated previously [30,32,33]. The methodological details of image analysis as a valid method for quantification have been described previously [32,34]. To obtain semi-quantitative data for IHC staining in follicular wall, at least five sections were evaluated for each specimen and antibody. The average density (% of immunopositive area) was calculated from at least 50 images of each area (granulosa and theca cells) in each section as a percentage of the total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain). These values were verified and normalized with controls carried across various runs using the same region (verified by image comparison) for calibration. The percentage of immunopositive area was calculated separately for each follicular compartment (granulosa and theca cells). Sections were analyzed with the observer blinded to the experimental group.

2.12. Statistical analysis

The statistical software package SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The distribution of data was tested for normality using the Kolmogorov-Smirnov test. Tests of homogeneity of variance between groups were conducted using Levene's test. The independent-Samples T test was used to evaluate the levels of changes between two groups. Differences in protein expression were assessed separately in each follicular layer. The results are expressed as mean ± SEM. A value of $p < 0.05$ was considered significant.

3. Results

3.1. NEFA, BHB, glucose, triacylglycerol and cholesterol concentrations

NEFA, BHB, glucose, triacylglycerol and cholesterol concentrations in plasma and follicular fluid from control and COD cows are shown in Fig. 1. Although systemic concentrations of NEFA and BHB in control and cystic cows were similar, the concentrations of NEFA and BHB in follicular fluid were higher in spontaneous cysts from cows with COD than in preovulatory follicles from control cows ($p < 0.05$). In addition, the intrafollicular concentrations of NEFA and BHB in control cows were lower than those detected in plasma and whole blood, respectively ($p < 0.05$). Even more, the intrafollicular concentration of BHB was higher than that in plasma from the COD animals ($p < 0.05$). The concentrations of glucose and triacylglycerol in follicular fluid were higher in preovulatory follicles than in spontaneous cysts ($p < 0.05$), whereas those in plasma were similar between groups ($p > 0.05$). In the control group, the systemic glucose concentration showed no differences from that detected in the follicular fluid ($p > 0.05$), whereas, in the COD group, it was higher in plasma than in follicular fluid ($p < 0.05$). Regarding triacylglycerol concentrations, both control and COD cows had similar systemic levels, with higher levels in follicular fluid from the control group than in that from the COD group ($p < 0.05$). In follicular fluid of spontaneous cysts, the cholesterol concentration was higher than in preovulatory follicles from control cows ($p < 0.05$). Moreover, cholesterol concentration was higher in plasma than in follicular fluid from both the control and COD groups ($p < 0.05$).

3.2. Insulin concentrations

Insulin concentrations in plasma and follicular fluid from control and COD cows are shown in Fig. 2. Insulin concentration in plasma was higher in preovulatory follicles from control cows than in spontaneous cysts from COD cows ($p < 0.05$). Insulin concentration in follicular fluid was similar between groups ($p > 0.05$),

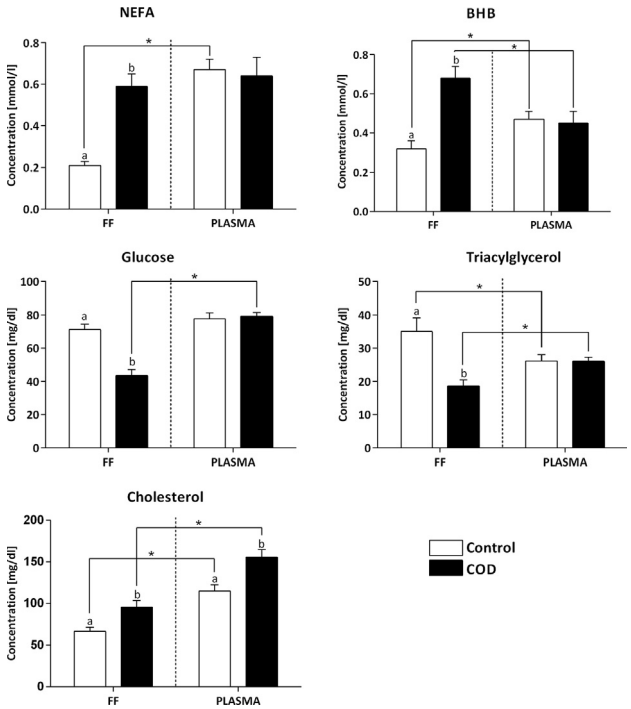

Fig. 1. NEFA, BHB, glucose, triacylglycerol and cholesterol concentration in plasma and follicular fluid of control (white bars) and cystic cows (black bars). Values represent mean ± SEM for each metabolite in plasma or follicular fluid. Bars with different letters are significantly different ($p < 0.05$). Bars with asterisk denote significant differences between plasma and follicular fluid within the same group ($p < 0.05$).

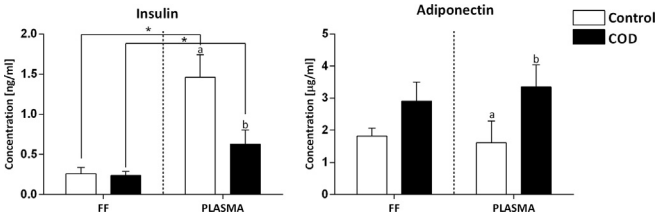

Fig. 2. Insulin and adiponectin concentration in plasma and follicular fluid of control (white bars) and cystic cows (black bars). Values represent mean ± SEM for each parameter in plasma or follicular fluid. Bars with different letters are significantly different ($p < 0.05$). Bars with asterisk denote significant differences between plasma and follicular fluid within the same group ($p < 0.05$).

whereas that in plasma was higher than in follicular fluid from the control and COD groups ($p < 0.05$).

3.3. Adiponectin concentrations

Adiponectin concentrations in plasma and follicular fluid from control and COD cows are shown in Fig. 2. Adiponectin concentration in follicular fluid was similar in control and COD cows ($p > 0.05$), whereas that in plasma was significantly higher in the COD group than in the control group ($p < 0.05$).

3.4. Protein expression

Protein expressions of AdipoR1, AdipoR2, AMPK, CPT1 and ACOX1 were determined in follicles at different developmental stages. All these proteins were detected in the cytoplasm of granulosa and theca interna cells of all the follicles analyzed (Figs. 3 and 4).

3.4.1. AdipoR1, AdipoR2 and AMPK protein expression

In most follicular categories, AdipoR1 protein expression was similar in both theca and granulosa cells from control cows and cows with COD ($p > 0.05$). However, AdipoR1 expression in theca cells was higher in atretic follicles from control cows than in those from cows with COD ($p < 0.05$). AdipoR2 protein expression in granulosa cells was similar in all the follicular structures evaluated from both control cows and cows with COD ($p > 0.05$), whereas that in theca cells was higher in follicular cysts than in control antral follicles (as reference structures) ($p < 0.05$). AMPK protein expression in granulosa cells was higher in follicular cysts than in control antral follicles (as reference structures) ($p < 0.05$), whereas that in theca cells was higher in atretic follicles from cows with COD than in those from control cows ($p < 0.05$) (Figs. 5 and 6).

3.4.2. CPT1 and ACOX1 protein expression

CPT1 protein expression in granulosa cells was higher in follicular cysts than in control antral follicles ($p < 0.05$), whereas that in theca cells was similar in all the follicular structures evaluated from control cows and cows with COD ($p > 0.05$).

ACOX1 protein expression in granulosa cells was similar in all the follicular structures evaluated from control cows and cows with COD ($p > 0.05$), whereas that in theca cells was higher in follicular cysts than in control antral follicles ($p < 0.05$). Also, ACOX1 protein expression in theca cells was higher in atretic follicles from cows with COD than in those from control cows ($p < 0.05$).

4. Discussion

The increasing demand in milk production has consequences on the reproduction rates of dairy cows, with important economic

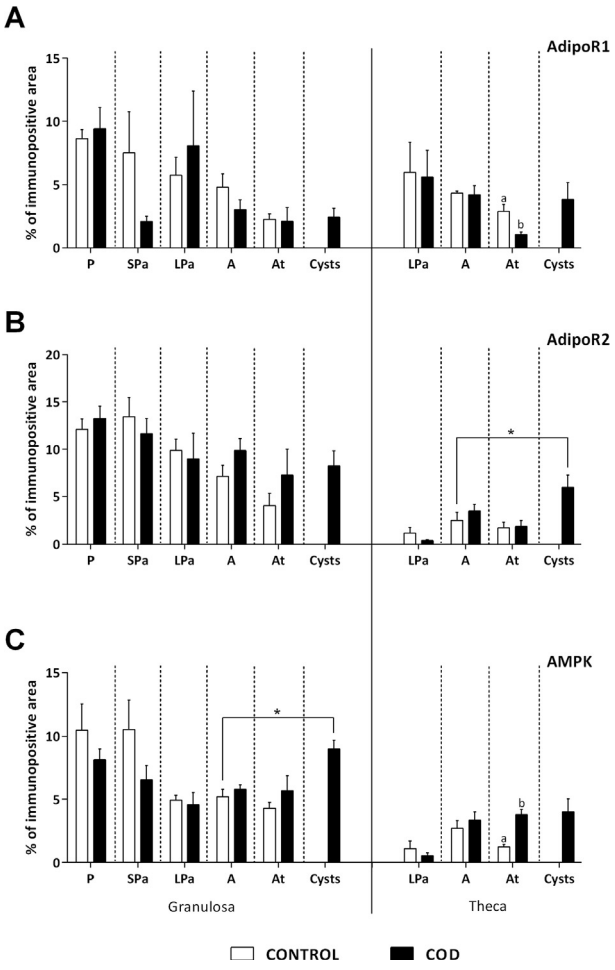

Fig. 3. Relative protein expression (measured as percentage of immunopositive area) of AdipoR1 (A), AdipoR2 (B) and AMPK (C) in granulosa and theca cells of primary follicles (P), small preantral follicles (SPa), large preantral follicles (LPa), antral follicles (A), atretic follicles (At) and follicular cysts (Cysts), in control cows (white bars) and cows with spontaneous COD (black bars). Values represent mean ± SEM. Bars with asterisk denote significant differences relative to the expression in follicular cysts in relation to control antral follicles ($p < 0.05$).

losses [35,36]. Previously, we have shown alterations in insulin signaling pathways in animals with COD [28]. These pathways have important effects on reproduction functions and are regulated by several molecules including metabolites (glucose, fatty acids, etc.) and other hormones (adiponectin, ghrelin, etc.), which act through energy sensors (AMPK, PPARs, etc.) [5,14,37]. In the present study, despite the similar plasma values in most of the metabolites analyzed, in follicular fluid of COD animals we detected lower glucose and triacylglycerol concentrations along with higher NEFA, cholesterol and BHB concentrations. Glucose plays an essential role in ovarian metabolism, being its main source of energy [38–40]. The main source of glucose in the follicular fluid is probably blood, so the lower glucose concentration found in cystic follicles compared to the controls and the lower concentration in follicular fluid related to that in plasma in cows with COD are probably due to a greater anaerobic glycolysis [40,41]. Furthermore, since VLDL-triacylglycerol does not pass through the follicular wall [40,42], the higher levels of NEFA found in follicular fluid of cystic follicles could be a consequence of triacylglycerol hydrolysis, and its availability would favor its oxidation to obtain energy. Moreover, in cystic follicles, we detected higher protein expression of CPT1 and ACOX1, two key enzymes involved in mitochondrial and

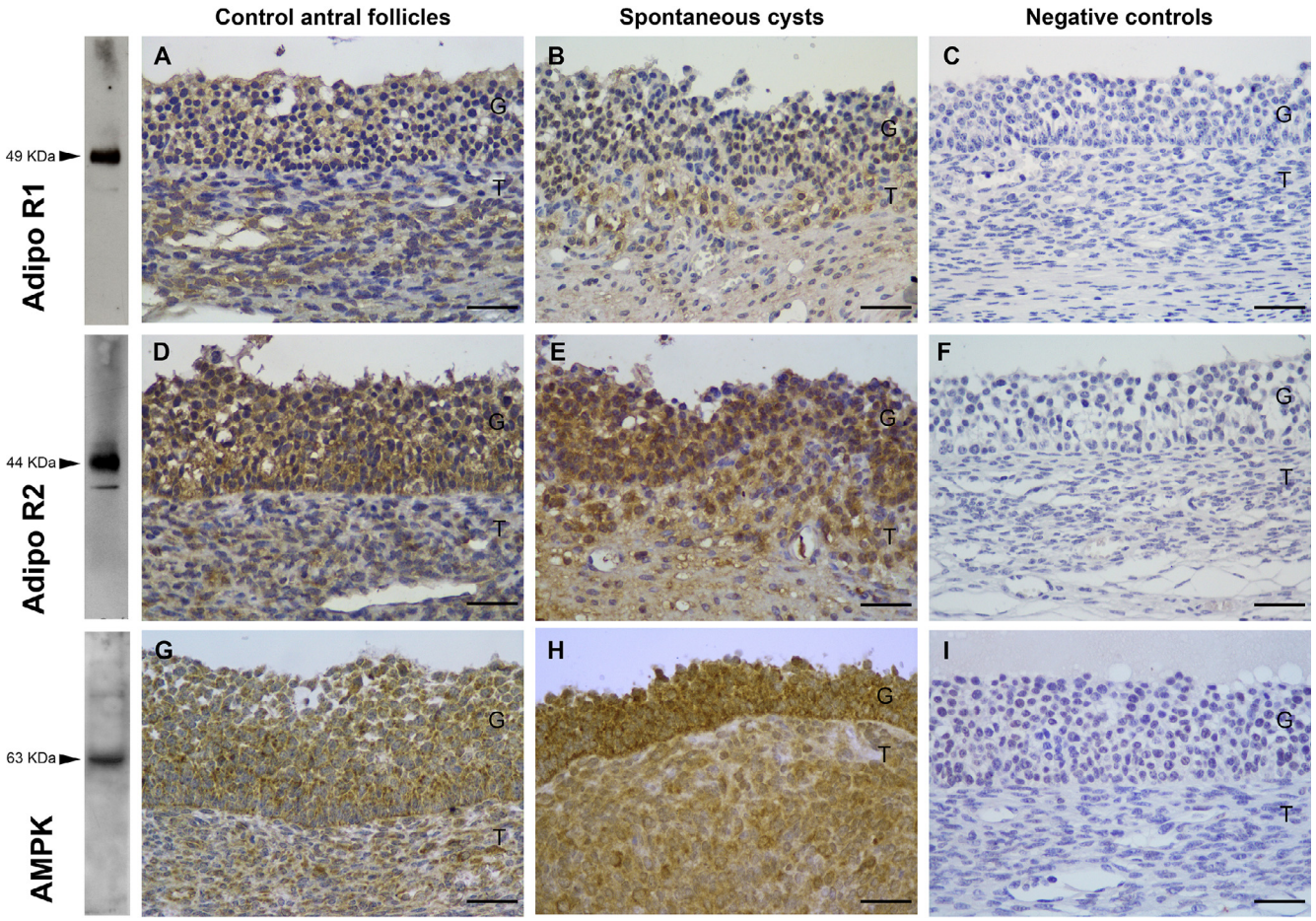

Fig. 4. Representative images of AdipoR1 (A, B), AdipoR2 (D, E) and AMPK (G, H) protein localization by immunohistochemistry in bovine follicles. Images represent control antral follicles (A, D and G), cystic follicles (B, E and H) and negative controls (C, F and I). Verification of antibody specificity by western blot analysis in an ovarian homogenate is shown in the left. T: theca cells, G: granulosa cells. Bars = 25 μm.

peroxisomal beta-oxidation respectively. In this sense, Elis et al. [43] have demonstrated the importance of fatty acid oxidation and synthesis in granulosa cells of cows for sustaining follicular growth. On the other hand, the increase in BHB and cholesterol concentrations in follicular fluid of cysts could be related to the higher availability of acetyl-CoA from the increased fatty acid oxidation [44]. Also, the systemic cholesterol concentration could contribute to its increase in the follicular fluid of cysts.

Previous studies have shown the harmful effects of NEFA on ovarian functionality, such as inhibition of bovine granulosa cell survival and proliferation, steroidogenesis, and follicular development and alteration in oocyte development [36,45,46]. Therefore, the higher levels of NEFA found in the follicular fluid of cysts could be altering the normal functionality of the cells, and could thus be affecting steroidogenesis. It has been reported that increased NEFA concentrations may have a putative direct toxic effect at the ovarian level [47]. The granulosa cell layer is a non-vascular compartment surrounded by follicular fluid and the basal membrane, whereas the theca interna cell layer is rich in blood capillaries. In agreement with that found by Leroy et al. [35], in the present study, NEFA concentrations were lower in the follicular fluid of dominant follicles than in blood serum, which indicates that granulosa cells might be exposed to lower NEFA concentrations than theca cells. However, NEFA metabolism is different in both compartments, with granulosa cells having a higher sensitivity to NEFA than theca cells [45]. In addition, in cows with COD, we detected similar NEFA concentrations in plasma and follicular fluid of cysts. NEFA, especially saturated long-chain fatty acids, seem to affect the reproductive physiology adversely. When granulosa and theca cells are incubated with high NEFA concentrations, their viability and steroidogenic capacity are hampered [4,45]. In this sense, we have previously detected altered expression of steroidogenic enzymes [23] and steroid hormone concentrations [22,23] in serum and follicular fluid of cows with COD, which could be related to altered NEFA concentrations, as reported in the present study. The lower progesterone concentration detected in cows with COD is in agreement with the *in vitro* study reported by Vanholder et al. [4] and could negatively modulate the ovarian functionality. On the other hand, we herein corroborated that cows with COD had lower plasma insulin levels than controls, and, as we have previously shown, this was accompanied by lower protein expression of some components of the insulin signaling cascade [28]. Many authors have demonstrated that the actions of insulin in the ovary, including steroidogenesis, growth of granulosa and theca cells [48,49], follicular maturation [50,51] and normal postpartum ovarian function [52], occur through the increased steroidogenic capacity of the dominant follicle [53]. Furthermore, it has been demonstrated that alterations in insulin concentrations can affect the development and maturation of follicles and the response to stimulation by LH, which can lead to anovulation and cyst formation [54]. Additionally, *in vitro* studies with bovine theca cells have shown that adiponectin decreases the androgen and progesterone

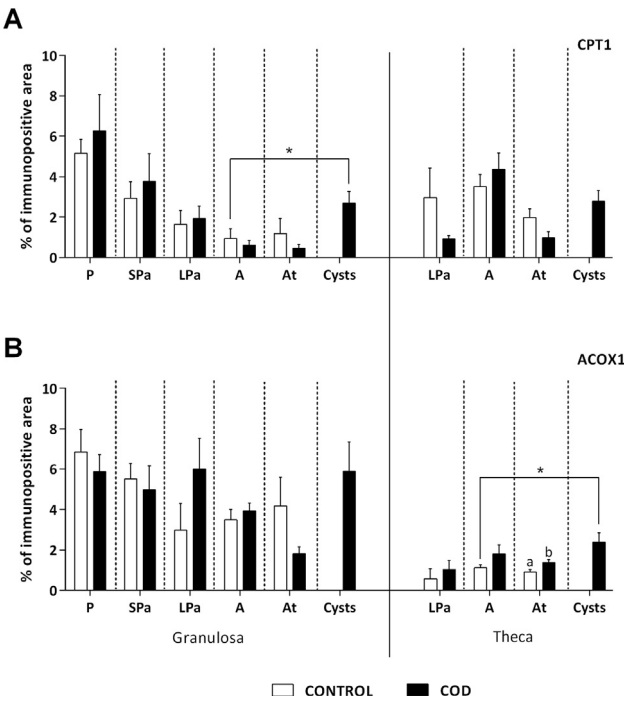

Fig. 5. Relative protein expression (measured as percentage of immunopositive area) of CPT1 (A) and ACOX1 (B) in granulosa and theca cells of primary follicles (P), small preantral follicles (SPa), large preantral follicles (LPa), antral follicles (A), atretic follicles (At) and follicular cysts (Cysts), in control cows (white bars) and cows with spontaneous COD (black bars). Values represent mean ± SEM. Bars with asterisk denote significant differences relative to the expression in follicular cysts in relation to control antral follicles ($p < 0.05$).

production induced by insulin [55]. Even more, different mRNA levels of adiponectin and adiponectin receptors have been detected in granulosa and theca cells of dairy cows at different follicular stages, suggesting that adiponectin may exert an important physiological role [56]. In granulosa cells, the growth of bovine healthy follicles was correlated with an increase in the gene expression of adiponectin and its receptors, whereas in theca cells, adiponectin expression decreased across follicular development [56]. Moreover, adiponectin has been reported to have suppressive effects on progesterone and androstenedione production and LH receptor expression in theca cells of bovine large follicles, probably regulated by LH and IGF-1 [57], which could be related to the lower concentrations of progesterone detected in cystic follicles [22,23]. Also, adiponectin increases insulin sensitivity and consequently insulin would be negatively regulating the mRNA levels of AdipoR1 and AdipoR2 via activation of PI3K [19,58]. Although several studies have reported lower concentrations of adiponectin in women with polycystic ovary syndrome (PCOS), mainly associated with obesity [19,57,59,60], here we detected that cows with COD had higher concentrations of adiponectin than control cows. In addition, in a retrospective study carried out by our group, we observed that the body condition at the voluntary waiting period did not influence the incidence of cysts [61]. In cattle, the body condition is related to fertility, mainly in the peripartum period, when overfeeding cows lipomobilize their reserves, increasing the levels of NEFA and related to a negative energy balance [62,63]. Furthermore, Ohtani et al. [64] measured serum adiponectin in cows at different stages of lactation, and observed an increase in postpartum lactation, probably associated with the energy deficit occurring during this period. In several species, the action of adiponectin has been studied in tissues such as liver and skeletal muscle, and it is known that this adipocytokine is involved in homeostasis and glucose and lipid metabolism, and participates in the control of reproductive functions [55,65–67]. In the present study, we found an increase in AdipoR2 and AMPK expression in theca and granulosa cells, respectively. Probably, the increased binding of adiponectin to its receptor favors glucose metabolism through AMPK [66]. Furthermore, PPAR-stimulated adiponectin expression would increase fatty acid oxidation and energy consumption [19,68], which could be related to the higher protein expression of CPT1 and ACOX1 in cows with COD detected in our study. Adiponectin, AMPK and PPARs may be key signals regulating the amount of energy required for the growth of follicles, oocytes, and embryos [14]. Therefore, the

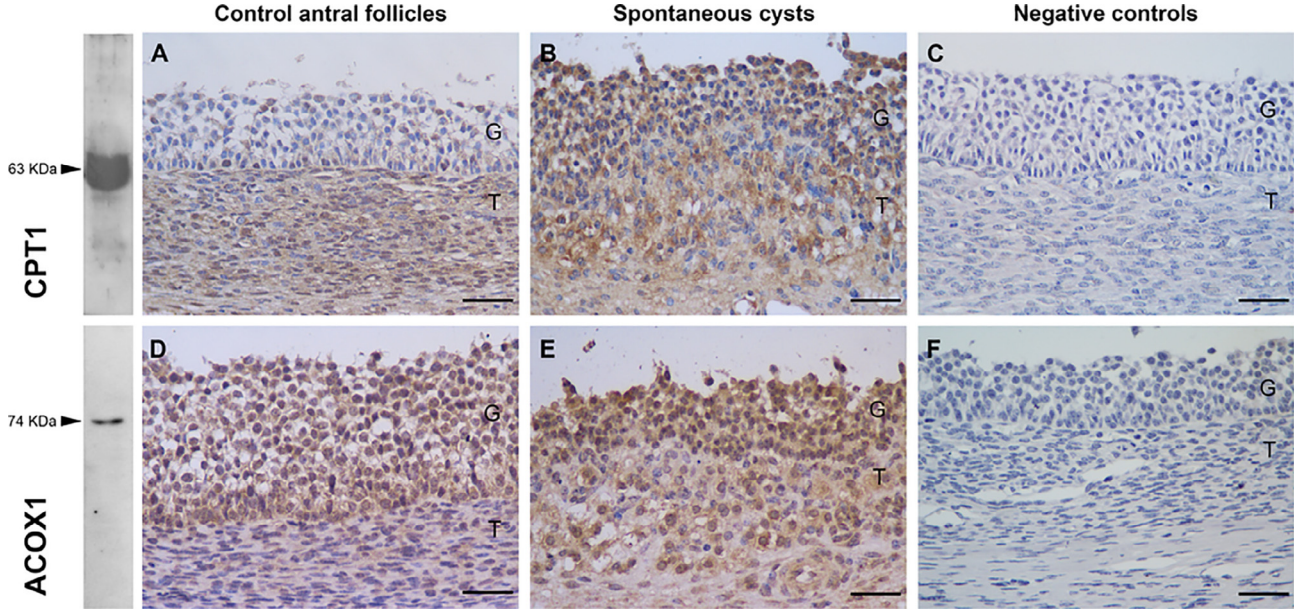

Fig. 6. Representative images of CPT1 (A, B) and ACOX1 (D, E) protein localization by immunohistochemistry in bovine follicles. Images represent control antral follicles (A and D), cystic follicles (B and E) and negative controls (C and F). Verification of antibody specificity by western blot analysis in an ovarian homogenate is shown in the left. T: theca cells, G: granulosa cells. Bars = 25 μm.

activation of these sensors could be related to the low intraovarian concentrations of glucose and triacylglycerol herein observed, as it has been widely studied in skeletal muscle and liver [19,68].

5. Conclusions

The results of the present study evidence a local alteration in some metabolic sensors in cystic follicles, which could promote fatty acid and glucose oxidation. Nevertheless, despite the increase in the oxidation of NEFA, their higher concentration in the follicular fluid of cystic follicles of cows with COD could be harmful for follicular cells, compromising normal ovarian folliculogenesis, including ovulation. In addition, they could generate an adverse microenvironment for the resumption of ovarian activity and could be a cause of the persistence of follicles and the recurrence of COD.

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