Upregulation of Glutathion S-Transferase mu 1 in Bovine Cystic Follicles

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ABSTRACT

Follicular cystic follicles (FCFs) show delayed regression with persistent follicle growth. However, the mechanism by which follicles are persistently grown remains unclear. Glutathione S-transferases (GSTs) are drug-metabolizing and detoxification enzymes that are involved in the intracellular transport and metabolism of steroid hormones. In this study, a proteomic analysis was performed to identify whether GST expression is changed in bovine FCFs and to predict the interactions between GST and other proteins. Normal follicles and FCFs were classified based on their sizes (5 to 10 mm and ≥25 mm). In bovine follicles, GST mu 1 (GSTM1) was detected as a differentially expressed protein (DEP) and significantly up-regulated in FCFs compared to normal follicles ($p<0.05$). Consistent with the proteomic results, semi-quantitative PCR data and western blot analysis revealed an up-regulation of GSTM1 in FCFs. Expression levels of aromatase and dehydrogenase proteins were changed in FCFs. These results show that the up-regulation of GSTM1 that is observed in bovine FCFs is likely to be responsible for the persistent follicle growth in FCFs as the activity of aromatase and the dehydrogenases.

(Key words : proteomic analysis, glutathione S-transferase, follicular cyst)

INTRODUCTION

Follicular cystic ovary (FCO) is a cause of reproductive failure in mammalian species. In FCO, affected follicles show delayed regression with persistent follicle growth and secretion of steroid hormones. These physiological changes could be produced by the extremely complex interactions between molecules, such as RPL15 and MAP1B (Choe *et al.*, 2010). Genetic and/or proteomic alterations affect the functioning of many kinds of cells (Hauptmann *et al.*, 2002, Osterberg *et al.*, 2006). Proteomic analysis is a powerful tool for the identification of proteins affected by a variety of factors which are modulated by microenvironment of FCO.

Glutathione S-transferases (GSTs) are a family of enzymes that catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophiles (Cho *et al.*, 2001). GSTs are drug-metabolizing and detoxification enzymes that are involved in the intracellular transport and metabolism of steroid hormones. The GST family of enzymes is composed of many cytosolic, mitochondrial, and microsomal proteins and account for up to 10% of the total cytosolic proteins in some mammalian organs (Boyer, 1989). Eight distinct classes of the soluble cytoplasmic mammalian GST have been identified as alpha (α), kappa (κ), mu (μ), omega (ω), pi (π), sigma (σ), theta (θ), and zeta (ζ). Of the GSTs, GST π was shown to be abundantly expressed in the stroma of human ovaries, and GST α was shown to be localized in the steroid-producing cells (Rahilly *et al.*, 1991).

Based on these reports, we performed a proteomic analysis to identify whether GSTs, which are likely to be related to ovarian pathophysiology, are changed in bovine FCO and to predict the interaction between GST and other proteins. In this study, we demonstrated that GST mu 1 (GSTM1) was up-regulated in follicular cystic follicles (FCFs) compared to normal follicles. Expression levels of aromatase and dehydrogenase proteins were changed in FCFs. The results were validated using semiquantitative PCR and western blot analysis.

MATERIALS AND METHODS

1. Samples

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Bovine ovaries at follicular phase were collected from a slaughterhouse and transported to the laboratory within 2 hrs on ice, either in RNA*later* solution (Qiagen, GmbH, Hilden, Germany), in ice-cold phosphate buffered saline (PBS), or in 37°C pre-warmed PBS containing 100 IU/ml of penicillin and 0.1 mg/ml streptomycin. Ovaries with follicles greater than 25 mm in diameter were used in this study (n=120). Normal and cystic follicles were isolated from same ovary and repetitions of experiments were performed with samples from different ovaries. Samples of the same group were pooled together. Ovaries with follicles greater than 25 mm in diameter in the absence of a corpus luteum in both the right and left ovaries were classified as FCOs. Follicular cysts were diagnosed based on macroscopic and endocrinological characteristics (Kesler *et al.*, 1981). The follicular walls of the cysts were thin and translucent (Isobe and Yoshimura, 2007). The granulosa cell layers were examined in the FCOs used in this study. All experiments were performed with the approval of the Animal Ethics Committee of Gyeongsang National University. Samples of perifollicular regions, including large follicles were cut with a razor blade and used for the preparation of RNA or protein. Follicular fluid (FF) was carefully aspirated from cystic and non- cystic healthy follicles with a 10 ml syringe fitted with an 18 or 23 gauge needle. Each pellet obtained from FF of normal follicles and FCFs was added to follicle samples of the same group.

2. Two-dimensional Electrophoresis and Image Analysis

For two-dimensional electrophoresis (2-DE), proteins were isolated using the ReadyPrep Protein Extraction Kit (Soluble/ Insoluble) according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Solubilized proteins (60 μg) isolated from bovine normal follicles and FCFs were mixed with a rehydration solution, containing 8 M/L urea, 4% 3-[(3-cholamidopropyl) dimethylammio]-1-propane-sulfonate (CHAPS), 10 mM/l dithiothreitol (DTT), and 0.2% carrier ampholytes (desired pH values), and applied to immobilized pH gradient (IPG) strips (ReadyStrip, Bio-Rad) in a re-swelling tray (Bio- Rad). The 17 cm-long strip gels with pH gradients ranging of 7 to 10 were used for a sharper separation. The protein samples were rehydrated on dried IPG strips, and the proteins were separated by isoelectric focusing (IEF) using a Protein IEF Cell (Bio-Rad) at 250 V for 15 min, 10,000 V for 3 h, and then held at 10,000 V for a total of 90,000 Vh for the 17 cm-long strip. Following IEF, the strips were equilibrated with 0.395 M/L of Tris buffer (pH 8.8) containing 6 M/L urea, 2% SDS, 2% DTT, 20% glycerol, and 0.01% bromophenol blue for 20 min. The strips were equilibrated again with the same buffer containing 2.5% iodoacetamide instead of DTT. The proteins were separated in the second dimension by SDS-PAGE gel (12.5% polyacrylamide) electrophoresis. The resolved protein spots on the gels were visualized by silver staining. After staining, the 2-DE gels (n=4, each pool) were scanned in visible light at a resolution of 254 dots per inch using a Fluor-S MultiImager (Bio-Rad). The image data was analyzed using version 8.0 of the PDQuest 2-DE Gel Analysis Software. The background values were subtracted from the gel images. After automatic spot detection and matching, manual editing was performed on all spots to eliminate any artifact dots that had been matched incorrectly by the software. The normalized intensities of the individual protein spot were calculated as the intensities of a particular spot from the intensities of all the spots of the gel, and are expressed as PPM. The differentially expressed protein spots were compared to average normal quantities of gels from each sample.

3. Destaining and In-gel Digestion of Protein Spots

The differentially expressed silver-stained spots in each sample were excised from the 2-DE gels and transferred into microcentrifuge tubes. The spots were destained with fresh chemical reducers in a 1:1 ratio of 30 mM/L potassium ferricyanide and 100 mM/L sodium thiosulfate until the brownish color of the spots disappeared. After destaining, the gel pieces were rinsed three times with distilled water to stop the destaining reaction, covered by ammonium bicarbonate (500 μl of 200 mM/l) for 30 min, dehydrated with 100 μl of acetonitrile (ACN), and then dried in a vacuum centrifuge. The gel pieces were rehydrated by covering them with a digestion buffer (50 mM/l NH_4HCO_3 and 5 mM/l $CaCl_2$) containing trypsin (12.5 ng/ml), and then incubated on ice for 45 min. The trypsin-containing digestion buffer covering gels were replaced with 20 μl of a digestion buffer without trypsin. The gels with digestion buffer were incubated overnight at 37°C. The gel pieces were vigorously vortexed for 30 min after adding 20 μl ACN. The supernatants were transferred into clean microcentrifuge tubes and dried in a vacuum centrifuge. The resulting pellets were dissolved in 2 μl of 0.1% trifluoroacetic acid (TFA).

4. Matrix-assisted Laser Desorption Ionization-time of Flight-mass Spectrometry (MALDI-TOF-MS)

For the matrix solution, α-cyano-4-hydroxycinnamic acid

(40 mg/ml) was dissolved in 50% ACN and 0.1% TFA. The matrix and sample solutions (1 μl each) were mixed and loaded into the target wells, and then dried for 10 min at room temperature. After drying, the well was washed using de-ionized water and subjected to MALDI-TOF-MS analysis using a Voyager Biospectrometry Workstation (PE Biosystems, Foster City, CA, USA) with the following parameters: 20 kV accelerating voltage, 75% grid voltage, 0.02% guide wire voltage, 150 ns delay, and a mass gate from 800 to 3,500. The peptide mass fingerprints were analyzed using the program MS-Fit of ProteinProspect, developed by the UCSF Mass Spectrometry Facility (http://prospector.ucsf.edu). The NCBI and Swiss-Prot database of *Bos taurus* proteins was searched to identify the proteins, using monoisotopic peptide masses and allowing a molecular-mass range of 2-DE ± 15%, a peptide mass accuracy of 50 p.p.m., and one partial cleavage.

5. Western Blot Analysis

Bovine follicles were homogenized in lysis buffer (RIPA buffer, Cell Signaling Technology, Danvers, MA, USA; 20 mM Tris-HCl (pH 7.5), 150 mM NaCl/1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin), incubated at 4℃ for 30 min, and centrifuged at 13,000 rpm (16,609 × g, Micro 17TR, Hanil, Korea) for 30 min at 4℃. Total protein in supernatants was measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Supernatant protein (50 μg/lane) was separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (0.45 μm, Millipore, Bedford, MA, USA) in a buffer solution (TBS; 25 mM Tris-base, 190 mM glycine, and 20% methanol) using a semi-dry blotter (Bio-Rad). The transferred blot was stained with Ponceau S solution to check for effective homogeneous transfer. Destained blots were blocked with 5% fat-free milk and 0.05% Tween 20 in TBS for 1 h and the membranes were immunoblotted with GSTM1 polyclonal antibodies (abcam, Cambridge, MA, USA and Santa Cruz Biotechnology, Inc., CA, USA) at 1:1,000 dilution at 4℃ overnight. After binding of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:3,000; Assay Designs, Ann Arbor, MI, USA) at room temperature for 1 h, antigens were detected by enhanced chemiluminescence (ECL Plus kit; ELPIS, Taejeon, Korea) according to the manufacturer's instructions.

6. Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The DEP expression level was confirmed by RT-PCR using gene-specific primer pair. The sequences of GSTM1 (GenBank accession number: BC102050) primers were 5'-GAAAAATTC AAGCTGGGCCT-3' (sense) and 5'-ACGGCAAGCTTCATGA ACAG-3' (antisense). Total RNA was extracted from bovine follicles with Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from the isolated follicular total RNA (3 μg) using oligo dT (SuperScript First-Strand Synthesis System, Invitrogen); it was subsequently used as a template for PCR amplification with *Taq* polymerase (Takara Bio Inc, Otsu, Shiga, Japan). The first-strand cDNA was quantified using a spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE, USA). The quantified cDNA was used as a template for PCR amplification. The PCR steps included initial denaturation at 94℃ for 5 min, then 30 cycles at 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 30 sec, and a final extension step at 72℃ for 10 min. The amplified PCR products were separated in 1.5% agarose gels stained with ethidium bromide. The bands were extracted and directly sequenced with an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems).

7. Data Analysis for Semi-quantitative PCR and Western Blot

LAS-4000 (Fujifilm Corp, Tokyo, Japan), a luminescent image analyzer, captured images of the agarose gels and western blots. The bands obtained from the RT-PCR and Western blot tests were quantified by Sigma Gel image analysis software (version 1.0, Jandel Scientific, CA, USA) or Quantity One software (version 4.6.3) attached to a GS-800 calibrated densitometer (Bio-Rad). Relative mRNA and protein levels were calculated by referring them to the amount of GAPDH and β-actin, respectively.

8. Statistics

Student's *t*-test was used with $p<0.05$ as the criterion for significance. Data are represented as mean ± SD.

RESULTS

1. Proteomic Analysis of the Bovine Cystic Follicles

The soluble proteins isolated from bovine follicles were proteomically analyzed to investigate differentially expressed proteins (DEPs) including GSH between normal follicles and FCFs.

The proteins were separated on isoelectric points (p*I*) 7~10 strips and SDS-PAGE. Representative 2-DE profiles of normal and FCFs are shown in Fig. 1. Approximately 50 protein spots per silver-stained gel were observed in p*I* 7~10 by the naked eye, and 16 spots were recorded by the PDQuest program. The numbers of protein spots observed by the naked eye were represented by counting strong spots. As shown in Fig. 1, 16 spots recorded by the PDQuest were selected and identified by peptide mass fingerprinting using MALDI-TOF-MS. The intensities of these 16 spots were changed in FCFs. The 15 observed proteins (No. 1 to 16) identified by MALDI-TOF-MS and database-searching were fibroleukin, cytochrome p450 19A1 (aromatase), guanylate cyclase soluble subunit alpha-1 (GCS-alpha-1), myoneurin, methylmalonate-semialdehyde dehydrogenase (MMSDH), growth/differentiating factor 9 (GDF-9), Vitrin, NADH dehydrogenase 1 alpha subcomplex subunit 1 (CI-MWFE), ligatin, GCS-alpha-1, Serine hydroxymethyltransferase (SHMT), UDP-glucose 6-dehydrogenase (UDP-Glc-dehydrogenase), Glutathione S-transferase mu 1 (GSTM1), electron transfer flavoprotein subunit alpha (Alpha-ETF), RNA-binding protein 7 (RNABP7), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Of these, nine proteins (fibroleukin, aromatase, MMSDH, GDF-9, vitrin, CI-MWFE, GSTM1, Alpha-ETF, and RNA-binding protein 7) were up-regulated, whereas six proteins (GCS-alpha-1, ligatin, myoneurin, SHMT, UDP-Glc-dehydrogenase, and GAPDH) were down-regulated in the FCFs. Table 1 summarized the up- and down-regulated proteins. GSTM1 (spot No. 13) showed a 25.5 kDa size and 7.3 p*I*. The differences in molecular weight (MW) and p*I* of DEPs identified in this study, especially GCS α-1 detected in two spots with different MW and p*I*, from MW and p*I* calculated from the reported primary structure may result from occurrence of post-transcription, translation modifications, or alternative splicing of transcripts (Fig. 1 and Table 1).

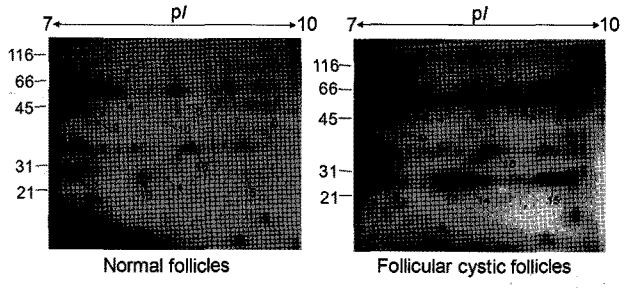

Fig. 1. Two-dimensional gel electrophoresis of bovine ovarian follicles. Representative two-dimensional gel electrophoresis profiles of normal and follicular cystic follicles. The soluble protein (60 μg) of each isolate was loaded on the 2-DE gel. The 2-DE gels were focused on 17-cm IPG strips with immobilized pH ranging 7 to 10. Proteins were then separated on 12.5% polyacrylamide gel. Molecular size markers are shown on the left-hand side of the gels. The proteins were visualized by silver staining. Number (1 to 16) represents DEP identified by PDQuest.

2. Confirmation by Semi-quantitative PCR and Western Blot Analysis

In the 2-DE analysis, GSTM1 was up-regulated. Fig. 2A shows enlarged images of GSTM1. In order to verify the 2-DE data, we performed western blot analysis with the antibodies against anti-GSTM1 purchased from two different companies (abcam and Santa Cruz). In agreement with 2-DE data, GSTM1 protein was significantly increased in the FCFs, compared to normal follicles (Fig. 2B). In order to validate GSTM1 protein identified in 2-DE analysis, semi-quantitative RT-PCR was performed using first-strand cDNA prepared from bovine follicles. Consistent with protein data, semi-quantitative PCR analysis showed an up-regulation of GSTM1 mRNA expression. The GSTM1 expression level was increased by 44% in cystic follicles compared to that in normal follicles (Fig. 2C).

DISCUSSION

This study reports for the first time that the expression level of GSTM1 is different between bovine normal follicles and FCFs. 2-DE proteomics, semi-quantitative PCR, and western blot analysis show fully consistent results of up-regulation of GSTM1 in FCFs.

A follicular cyst results from a follicle that grows without rupturing or from one that releases its oocyte due to dysfunctional hormonal signals. The granulosa and theca interna cells of the cystic follicles show weak proliferative activity and a low frequency of apoptosis; therefore, the cystic follicles grow slowly and delay follicular regression (Isobe and Yoshimura, 2007). Cystic follicles containing granulosa cells are distinguished by a high concentration of estrogen and low concentrations of progesterone and testosterone in their follicular fluids. Low concentrations of testosterone and high concentrations of estrogen may result from the action of aromatase, which converts testosterone to estrogen. In this study, aromatase was up-regulated in FCFs (Table 1). Estrogen is an endogenous hormone that is involved in many important cellular processes, such as cell proliferation, and increases the cell proliferation in estrogen receptor α positive cells (Tan *et al.*,

2009). GST isoforms are also known to be involved in the metabolism of steroid hormones (Rahilly *et al.*, 1991; Haas *et al.*, 2006).

GSTM1 belongs to a superfamily of GSTs that metabolizes a broad range of reactive oxygen species (ROS) and xenobiotics by conjugation with glutathione. GSTM1 polymorphisms have been linked to an increasing number of cancers (Hirvonen *et al.* 1993; Zhong *et al.* 1993). Knockdown of GSTM1

Table 1. List of differentially expressed proteins identified by MALDI-TOF-MS in bovine follicular cystic follicles

Spot No.	Protein names	Annotation	MW/pI	Swiss-Prot. Acc. Nos.	CV (%)	Fold change
1	Fibroleukin	Physiologic lymphocyte functions at mucosal sites	50.7/7.5	Q29RY7	41.5/73.6	3.0 (△)
2	Cytochrome P450 19A1 (aromatase)	Catalyzes the formation of aromatic C18 estrogens from C19 androgens	58.1/8.0	P46194	35.5/67.1	5.3 (△)
3	Guanylate cyclase soluble subunit alpha-1 (GCS-alpha-1)	cGMP biosynthesis	77.5/8.4	P19687	21.3/80.7	−5.2 (▽)
4	Myoneurin	Transcription regulation	65.4/8.6	Q3B7N9	24.3/80.7	−31.2 (▽)
5	Methylmalonate-semialdehyde dehydrogenase, mitochondrial (MMSDH)	Valine and pyrimidine metabolism	58.1/8.3	Q07536	15.4/71.1	5.1 (△)
6	Growth/differentiation factor 9 (GDF-9)	Ovarian folliculogenesis	51.9/9.0	Q9GK68	64.5/116.6	6.8 (△)
7	Vitrin	Promotes matrix assembly and cell adhesiveness	70.9/9.0	Q95LI2	25.9/58.5	4.2 (△)
8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1 (CI-MWFE)	Immediate electron acceptor for the enzyme	81.1/9.6	Q02377	38.0/84.6	4.7 (△)
9	Ligatin	Trafficking receptor for phosphoglycoproteins	63.6/8.4	Q58CR3	20.5/80.7	−11.0 (▽)
10	GCS-alpha-1	cGMP biosynthesis	77.5/8.4	P19687	32.0/80.7	−5.0 (▽)
11	Serine hydroxymethyltransferase, cytosolic (SHMT)	Interconversion of serine and glycine	52.8/8.3	Q5E9P9	17.0/92.4	−2.7 (▽)
12	UDP-glucose 6-dehydrogenase (UDP-Glc dehydrogenase)	Biosynthesis of glycosaminoglycans	55.1/7.5	P12378	8.5/82.5	−3.9 (▽)
13	Glutathione S-transferase mu 1 (GSTM1)	Protection against the thiol-mediated metal-catalysed oxidative inactivation of enzymes	25.5/7.3	Q9N0V4	24.2/101.5	2.0 (△)
14	Electron transfer flavoprotein subunit alpha, mitochondrial (Alpha-ETF)	A specific electron acceptor for several dehydrogenases	35.0/8.8	Q2KJE4	53.8/77.2	75.8 (△)
15	RNA-binding protein 7	Meiosis	30.0/9.7	Q3MHY8	60.7/70.3	23.0 (△)
16	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Glycolysis	35.8/8.5	P10096	24.7/33.9	−2.4 (▽)

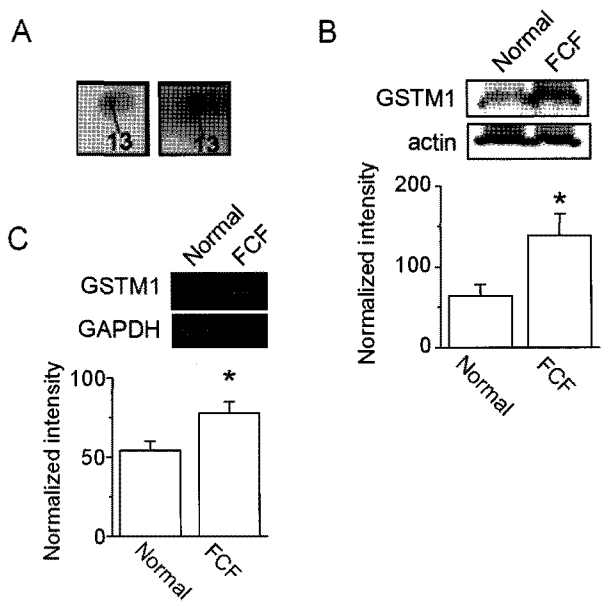

Fig. 2. Validation of 2-DE analysis by semi-quantitative PCR and western blotting in normal and follicular cystic follicles. (A) The enlarged images of GSTM1 spots obtained from Fig. 1. (B) Western blot analyses of GSTM1 protein. The bar graph shows the up-regulation of GSTM1 in FCF. Each bar represents the mean ± SD of three repeated experiments. Asterisks indicate the significant difference from normal follicles ($p<0.05$). (C) RT-PCR products for GSTM1 (497 bp) derived from bovine follicles. The expression levels were normalized to GAPDH. Each bar represents mean ± SD of four repeated experiments. Asterisks indicate the significant difference from normal follicles ($p<0.05$). FCF represents follicular cystic follicles.

by small interfering RNA resulted in an increase in the proliferation and migration of vascular smooth muscle cells and increased ROS levels (Yang *et al.* 2009). In contrast, knockdown of GST-pi resulted in significant decreases in the proliferation rate in androgen-independent prostate cancer cells (Hokaiwado *et al.*, 2008). Mouse GSTM1 (mGSTM1) inhibits apoptosis signal-regulating kinase 1 (ASK1)-dependent apoptotic cell death (Cho *et al.*, 2001). ASK1 plays a critical role in cytokine- and stress-induced apoptosis and interacts with mGSTM1. The observed up-regulation of GSTM1 in the FCFs with weak proliferative activity and a low frequency of apoptosis in our study suggest that GSTM1 might be involved in the proliferation of granulosa and theca interna cells.

In addition to the activities of the GST, dehydrogenase activities are also implicated in the defense against oxidative stress. Dehydrogenases are enzymes that oxidize a substrate by transferring one or more hydrides to an acceptor. In our study, changes in the dehydrogenase proteins (MMSDH, CI-MWFE, UDP-Glc-dehydrogenase, Alpha-ETF, and GAPDH) were detected in the FCFs. GST activity was noncompetitively inhibited by acyl-CoA and PEG-carboxylate-CoA, which suggests the interaction of the GSTs with the dehydrogenases buffers the toxicity of PEG-carboxylate-CoA (Somyoonsap *et al.*, 2008).

In conclusion, these results show that in addition to aromatase and dehydrogenases, the up-regulation of GSTM1 that is observed in bovine FCFs is likely to be responsible for persistent follicle growth in FCFs.

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