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Hormonal regulation of sirtuins activity and expression in bovine granulosa cells

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Abstract

Sirtuins (SIRTs) are a family of seven NAD+-dependent histone deacetylases that regulate several biological reactions. How SIRTs regulate ovarian steroidogenesis in cattle remains to be fully unveiled. We hypothesize that SIRTs expression and activity are regulated by hormones that influence steroidogenesis in bovine granulosa cells (GC). Bovine ovaries were collected at a commercial slaughterhouse and GC were isolated from small antral follicles (1-5 mm on surface diameter). Cells were treated with hormones that regulate ovarian folliculogenesis: FSH, IGF1, fibroblast growth factor (FGF) 2, FGF9, and their combinations. Cells were cultured for total RNA isolation (n=6 pools) with miRNeasy microkit (Qiagen) or for isolation of nuclear and cytoplasmic extracts (n=3 pools) with EpiQuik Nuclear Extraction Kit (Epigentek) according to the manufacturers' instructions. Relative mRNA abundance was quantified via qPCR whereas SIRTs activity in cytoplasmic (SIRTs 1, 6, and 7) and nuclear (SIRTs 2, 4, and 5) extracts was analyzed with the Epigenase Universal SIRT Activity/Inhibition assay kit. Data were analyzed via ANOVA with GLM procedures of SAS. In terms of mRNA relative abundance, FSH+IGF1+FGF9 increased mRNA relative expression of SIRTs 2 to 7 in comparison to negative control and of SIRTs 2, 3, 4, 6, and 7 in comparison to FSH+IGF1; FSH+IGF1+FGF2 increased mRNA relative abundance of SIRTs 2 and 6 in comparison to FSH+IGF1; FGF2 alone increased SIRT1 in comparison to negative control ($p < 0.05$). In term of SIRTs activity, FGF2 alone increased nuclear SIRTs activity in comparison to FSH, IGF1, FSH+IGF1, and FGF9 alone; FSH+IGF1+FGF2 increased cytoplasmic SIRTs activity in comparison to all treatments ($p < 0.05$). Taken together, our data demonstrate that SIRTs expression and activity in bovine GC are regulated by hormones that influence steroidogenesis.

Life has taught me to never give up
Neither to win, nor to lose, but to look forward inner evolution
They can take out everything I own
But they can't erase the good things I did

Charlie Brown Jr

To my parents, for their love and strength.

To my brother, for being always on my side.

To my grandfather and cousin,

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I dedicate

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INTRODUCTION

Ovarian steroidogenesis is a crucial process in the maintenance and regulation of the female mammalian reproductive tract. It is important for follicle growth, oocyte maturation, and fertility 2006). Estradiol is primarily synthesized within the follicles of the ovaries by ovarian steroidogenesis (Miller & Auchus, 2011). Estradiol is essential for several functions such as: maturation of the oocyte, development of female secondary sexual characteristics, fertility, and health (Miller & Auchus, 2011)Despite that ovarian steroidogenesis is well described in the literature and has been researched for many years, much is unknown about this elaborate process.

Sirtuins (SIRTs) are enzymes that can be found in almost all domains of life and they act as mediators in several cellular pathways (Chang & Guarente, 2014). SIRTs are defined as class III histone deacetylases dependent of NAD+. They are a family that consists of seven members, 1 – 7 (Michan & Sinclair, 2007). SIRT1, SIRT3, and SIRT5 have been reported to act in the ovaries by mediating several biological processes, including ovarian steroidogenesis and some diseases such as polycystic ovarian syndrome (Dong et al., 2016; Tao et al., 2015). In addition, SIRTs interact with various hormones in the mammalian female (Sirotkin et al., 2020; Tao et al., 2015; Zhang et al., 2016). Therefore, a comprehensive study investigating ovarian steroidogenesis is important for the understanding of molecular regulation of ovarian steroidogenesis. We hypothesized that factors that regulate ovarian steroidogenesis and granulosa cell differentiation influence sirtuins gene expression and activity.

In this study, we used bovine granulosa cells to investigate the association between steroidogenesis and sirtuins expression and activity in a mammalian mono-ovulatory species. Different hormones related in the processes of ovarian steroidogenesis and follicular differentiation were used to treat bovine granulosa cells in vitro.

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Chapter I

Review of Literature

1. Ovarian Steroidogenesis

1.1 Introduction

Steroidogenesis is defined as the production of steroids and was first reported in 1930s (Kendall, 1934; Steiger & Reichstein, 1937). It is the biological process by which steroids are generated from cholesterol and changed into other steroids (Hanukoglu, 1992). The major classes of steroid hormones are the progestogen, corticoids, androgens, and estrogens (Miller & Auchus, 2011). Sex steroids such as androgens, progestogens, and estrogens are critically important to regulate a plethora of physiological mechanisms, including sexual differentiation and development, secondary sex characteristics, reproductive physiology, and energy metabolism (Couse & Korach, 1998; Faulds et al., 2012; Stocco & Clark, 1996).

The cholesterol used for synthesis of steroids in the cells is derived either from plasma lipoproteins or from de novo cholesterol biosynthesis from acetate (Hsueh et al., 1994; Hu et al., 2010). The de novo cholesterol biosynthesis involves the conversion of acetate through a series of complex enzymatic steps and is dependent on the activity of the rate-limiting enzyme 3-hydroxy-3-methyl coenzyme A reductase whereas the synthesis of steroids from lipoproteins is regulated by the availability of lipoproteins and the lipoprotein receptor-dependent uptake system (Hsueh et al., 1984; Hu et al., 2010).

1.2 Process of ovarian steroidogenesis

In the ovary, the process of steroidogenesis is regulated by different compartments within the ovarian follicle (Hsueh et al., 1984). Ovarian steroidogenesis is not only regulated by intraovarian factors, but also by hormones arriving from various tissues, such as gonadotropins from the hypophysis (Hsueh & Erickson, 1979; Leung & Armstrongl, 1980; Spicer et al., 1993). Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are glycoproteins secreted from cells in the anterior pituitary and are called gonadotropins because they stimulate gonadal functions (Sherwood et al., 1993).

The main regulator of LH and FSH is the gonadotropin-releasing hormone (GnRH), an amino acid peptide that is synthesized and released from hypothalamic neurons and binds to receptors on gonadotropes in the anterior pituitary (Seeburg et al., 1987). When GnRH recognizes

and binds to the anterior pituitary gland, it stimulates the production of FSH and LH (Mcgee & Hsueh, 2000; Spicer & Echternkamp, 1986).

The understanding of ovarian steroidogenesis is complemented with the two cell, two-gonadotropin theory of ovarian steroidogenesis that explains how ovarian steroids are synthesized from cholesterol through cooperative actions of theca (TC) and granulosa cells (GC) (McNatty et al., 1979). The theory states that after the gonadotropin LH binds to luteinizing/chorionic gonadotropin receptor (LH/CGR) on TC to stimulate the expression of the steroidogenic enzymes required for androgens production. Androgens will serve as substrate for estradiol production in GC (Gibori & Sridaran, 1981). Then, the gonadotropin FSH binds to the follicle-stimulating hormone receptor (FSHR) in GC to stimulate estrogen synthesis (Stocco & Clark, 1996).

For the production of steroids, cholesterol is transported into the mitochondria by steroidogenic acute regulatory protein (StAR), which is expressed in ovarian TC and GC (Stocco & Clark, 1996). In the mitochondria, the cholesterol side-chain cleavage enzyme, commonly called P450scc and encoded by the gene *CYP11A1*, catalyzes the conversion of cholesterol to pregnenolone in a rate-limiting step of steroidogenesis (Miller, 1988). Pregnenolone is converted into progesterone by the enzyme 3b-hydroxysteroid dehydrogenase (*3bHSD*) (Canosa et al., 1998). Androgens are produced in TC through oxidation of progesterone and pregnenolone, which is catalyzed by the enzyme cytochrome P450 17A1 (*CYP17A1*) (Pallan et al., 2015). Androgens produced in TC will be converted into estrogens by the enzyme aromatase, encoded by the gene *CYP19A1*, in GC (Simoni et al., 1997). Aromatase is located at the endoplasmic reticulum and its expression is stimulated by FSH (Fournet-Dulguerovf et al., 1987; Ryan, 1982; Strauss & FitzGerald, 2019).

Estradiol (E2) is the main estrogen produced by the ovaries (Nelson & Bulun, 2001) and regulates several reproductive processes: is responsible for the regulation of estrous and female reproductive cycle (Sullivan & Wira, 1981); works in the development of secondary sexual attributes of females and has crucial importance to preserve and maintain female reproductive tissues such as the uterus, mammary glands and pregnancy (Ryan, 1982); regulates the maturation and release of the egg during ovulation (Lamy et al., 2016). Estradiol is synthesized primarily in the ovaries and levels decline as females age (Broekmans et al., 2009).

2. Histones Acetylation and Sirtuins

2.1 Introduction

Epigenetics refers to heritable changes in gene regulation without alterations of the genetic code itself (Ho & Burggren, 2010). The term epigenetics was defined by Waddington in 1942 and it was derived from the Greek word "epigenesis" which originally described the influence of genetic processes on development (Deichmann, 2016). Epigenetic changes occur naturally to regulate physiological mechanisms and can also be influenced by numerous factors including age, environment, and diet (Ledford, 2015). In addition, epigenetic changes can have detrimental effects that can result in diseases like cancer (Jovanovic et al., 2010; Mann, 2014; Natanzon et al., 2018; Sharma et al., 2010). Furthermore, epigenetic modifications can be inherited by future generations (Furuhashi et al., 2010; Skvortsova et al., 2018). To date, known epigenetic mechanisms include: DNA methylation, histone modifications, and non-coding RNA (ncRNA) (Tammen et al., 2013). Researchers are continuously uncovering the role of epigenetics in diverse areas and applications. In this literature review, we will explore histone modifications, specifically histones acetylation.

2.2 Histones Characterization

Histones are proteins found inside the nucleus of eukaryotic cells associated with DNA (Shechter & Allis, 2007). Their function is to package DNA into structural units called nucleosomes (Delange & Smith, 1971). There are five types of histones known to date: H1 (or H5), H2A, H2B, H3 and H4. H1 and its homologous protein H5 are involved in higher-order structures of chromatin and are known as linker histones whereas the other four types of histones associate with DNA to form nucleosomes and are known as core histones (Dhall & Chatterjee, 2015; Fyodorov et al., 2018).

The nucleosome constitutes the basic structural unit of chromatin and is composed of a nucleosome core, a linker DNA, and a linker histone (Cutter & Hayes, 2015). The nucleosome core is composed by an octamer of two copies of each core histone. Within the nucleosome core particle, each core histone forms a histone fold structure with a flexible amino-terminal tail (Fyodorov et al., 2018). The linker histone binds to the nucleosome core around the DNA entry and exit sites and protects the DNA linking adjacent nucleosomes (Biterge & Schneider, 2014).

2.3. Histone Modifications

Histones have crucial functions in the regulation of gene expression (Roth et al., 2001). In eukaryotes a histone modification is a post-translational modification to histone proteins and this modification can occur due to methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation (Wood & Shilatifard, 2004). Histone modifications affect interactions of histones with DNA and adjacent proteins (Biterge & Schneider, 2014) and can regulate gene expression by altering the chromatin structure or recruiting histone modifiers (Gelato & Fischle, 2008). Modification of histones can affect various biological processes, such as DNA damage/repair, chromosome packaging and transcriptional activation or inactivation (Bártová et al., 2008; Kreimeyer et al., 1984).

Histone acetylation is a key regulator of gene transcription, which influences gene expression and physiological activity (Sterner & Berger, 2000). Post-translational acetylation of histones is mediated by histone acetyltransferase (HAT) enzymes (Kuo & Allis, 1998). These enzymes acetylate lysine residues of histones via transferring of an acetyl group from acetyl-coenzyme (Acetyl-CoA) to the ε-NH_3 groups of lysine side chains within a histone's basic N-terminal tail region (Kuo & Allis, 1998; Struhl, 1998). This process of lysine acetylation neutralizes the positive charge of lysine residues in the tail of histones and leads to a loosening of the protein-DNA contacts, which increasing access of transcription factors and RNA polymerase to DNA and is associated with increased gene expression (Grunstein, 1997; Lee & Workman, 2007).

In contrast to histone acetyltransferases, the histone deacetylases (HDACs) catalyze the removal of the acetyl group from the acetyl lysine residue (Kuo & Allis, 1998). This process brings back the positive charge of the N-terminal tails of histones and increases their affinity for the DNA, leading to a repressive chromatin structure, displacing transcription factors from gene promoters and thereby repressing transcription (Kuo & Allis, 1998). Currently, there are two known families and four classes of mammalian HDACs. The first family is composed of zinc-dependent HDACs and comprises the classes I, II, and IV of HDACs (Ropero & Esteller, 2007; Yang & Seto, 2007). The second family is composed of NAD-dependent HDACs and comprises the Class IV of HDACs, also known as sirtuins (Dai & Faller, 2008).

2.4 Sirtuins: Class III HDACs

Sirtuins (SIRTs) are a HDAC NAD+-dependent and ADP-ribosylation family that consists of seven members (SIRT 1-7) and can be found in almost all domains of life (Michan & Sinclair, 2007). Of the seven members of sirtuins, SIRT4 has not yet been reported with deacetylase activity in vitro. However, a few studies demonstrated that SIRT4 is similar to other mitochondrial sirtuins and it has all the characteristics of a deacetylase enzyme (Anderson et al., 2017; Haigis & Guarente, 2006; Imai & Guarente, 2010).

Although the distinct roles of each of the seven SIRTs are still being fully unveiled, several studies have been unraveling their functions and how they act. Several studies report SIRTs as important mediators of cell metabolism and regulators of a large number of cellular pathways and is now well-established that SIRTs are related to a variety of biological processes such as: aging, cancer, central nervous system, immune system and metabolism (Chandramowlishwaran et al., 2020; de Matteis et al., 2019; Lee et al., 2019; Saunders & Verdin, 2007). The location of SIRTs within the cells varies (Table 1) and affects the way they target molecules and their biological roles (Kelly et al., 2010).

Table 1 – Sirtuins: their location and function

SIRTUIN	ACITIVITY	CELLULAR LOCATION
SIRT1	Deacetylation	Nucleus & Cytoplasm
SIRT2	Deacetylation & ADP-Ribosylation	Cytoplasm
SIRT3	Deacetylation	Mitochondria
SIRT4	ADP-Ribosylation	Mitochondria
SIRT5	Deacetylation	Mitochondria
SIRT6	Deacetylase & ADP-Ribosylation	Nucleus
SIRT7	Deacetylation	Nucleolus

2.5. Sirtuins and reproductive physiology

The expression of sirtuins in the ovary has been reported in several studies. SIRT1 is expressed in porcine ovarian follicles, epithelium, and stroma as well as in luteinized granulosa cells in rats. (Morita et al., 2012; Zhao et al., 2014). SIRT2 was reported in ovarian granulosa cells (GC), oocytes and theca cells in bovine (Xu et al., 2019). SIRT3 and SIRT5 have been detected in ovarian GC and cumulus oophorus surrounding the oocyte in humans (Pacella-Ince et al., 2014a,

2014b). SIRT4 and SIRT6 are active in primordial murine follicles, primary follicles, secondary follicles, antral follicles, pre-ovulatory follicles, and the corpus luteum (Kong et al., 2020). SIRT7 has been detected in human ovaries (Aljada, 2015). (Table 2).

Table 2 - Sirtuins gene expression reported in mammal ovaries.

SIRTUINS	LOCALIZATION OVARIES	REFERENCE
SIRT 1	Ovarian follicle, ovarian epithelium, and stroma, luteinized granulosa cells, primordial follicle	Zhao et al., 2014 Han et al., 2017
SIRT 2	Ovarian granulosa cells, oocytes and theca cells	Xu et al., 2019
SIRT 3	Ovarian granulosa cells, cumulus cells	Pacella-Ince et al., 2014b
SIRT 4	primordial follicles, primary follicles, secondary follicles, antral follicles, pre-ovulatory follicles, and corpus luteum	Kong et al., 2020
SIRT 5	Ovarian granulosa cells, cumulus cells	Pacella-Ince et al., 2014a
SIRT 6	Ovarian granulosa cell, follicular fluid, primordial follicles, primary follicles, secondary follicles, antral follicles, pre-ovulatory follicles, and the corpus luteum	Kong et al., 2020
SIRT 7	Ovaries and ovarian cancer	Kim et al., 2013 Aljada et al., 2015

SIRT1 is one of the most studied of the seven sirtuins due to its participation in different biological processes, including reproductive physiology. SIRT1 was demonstrated to stimulate GC proliferation and conserve ovarian primordial follicle pool in mice (Kong et al., 2020). SIRT1 in high levels of expression was associated with transformation of ovarian tissue with ovarian carcinoma (Mvunta et al., 2017). In contrast, SIRT1 was detected in very low levels in ovaries of

old mice and also mice treated with chemotherapy (Mvunta et al., 2017; Zhang et al., 2016). SIRT1 has been reported to regulate effects of vistafin, that is an adipocyte hormone on human GC steroidogenesis (Reverchon et al., 2016). Interestingly, SIRT1 expression can be influenced by hormones that directly affect ovarian function, such as FSH, insulin-like growth factor I (IGF-I), and oxytocin in porcine GC (Sirotkin et al., 2020).

Studies addressing the role of SIRT2 in reproduction are limited. In a recent in vitro study of bovine GC, inhibition of SIRT2 stimulated synthesis of progesterone and progesterone receptor while decreased secretion of estradiol and testosterone (Xu et al., 2019). Also, mRNA expression of *CYP17a1* and *STAR* (steroidogenic enzymes) were suppressed by SIRT2 inhibition whereas *CYP11A1* mRNA was increased by SIRT2 inhibition (Xu et al., 2019).

The SIRT3 expression varies in ovarian cells depending of their age and stage (Zhang et al., 2016). SIRT3 was detected at low levels in ovaries of old mice and also mice treated with chemotherapy and it was demonstrated that SIRT3 acts repressing ovarian cancer cell migration and invasion in vitro and suppressing metastasis of ovarian cancer in vivo (Dong et al., 2016). Therefore, SIRT3 is suggested to be a possible ovarian marker related to health and age. In addition, SIRT3 was reported to act improving the follicular environment via regulation of glutamate dehydrogenase and carbamoyl phosphate synthase I (Pacella-Ince et al., 2014a)

SIRT4 mRNA expression gradually declined in mice ovaries postnatal (1 to 23 days postpartum). This downregulation suggests that SIRT4 can be a marker of ovarian follicular development (Kong et al., 2020). SIRT4 has reduced expression in human ovarian cancer and when SIRT4 is knocked out, spontaneously development of tumors is observed, suggesting that SIRT4 may function repressing tumors in ovarian tissues (Fu et al., 2017).

SIRT5 expression is reduced in granulosa and cumulus cell of women with low ovarian reserve (Pacella-Ince et al., 2014b). In addition, SIRT5 mRNA expression has been reported in ovarian cancer (Bringman-Rodenbarger et al., 2018). SIRT6 expression has been associated with the quantity of primordial ovarian follicles and SIRT6 was reported at low expression levels in ovaries of old mice (Zhang et al., 2016). The amount of SIRT6 varies in ovarian cells depending of their age, stage and health (Zhang et al., 2016). Therefore, SIRT6 can be possibly a marker of ovarian reserve and ovarian aging. Also, SIRT6 when silenced due to aging or stress, loses its ability to silence LINE1 retrotransposons, leading to possible ovarian cancer (Aljada, 2015; Bringman-Rodenbarger et al., 2018; Van Meter et al., 2014).

Studies addressing the role of SIRT7 in ovarian physiology are limited. SIRT7 has been reported in ovarian cancer, indicating that SIRT7 could be used as a marker for ovarian cancer (Kim et al., 2017). Furthermore, exosomal miRNA-17-5p derived from human umbilical cord mesenchymal stem cells has been reported to restore ovarian phenotype and function in a premature ovarian insufficiency mouse model via inhibition of SIRT7 expression (Ding et al., 2020).

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Chapter II

Hormones regulating ovarian steroidogenesis influence sirtuins gene expression and activity in bovine granulosa cells

Introduction

Ovarian steroidogenesis is a crucial process in the maintenance and regulation of the female mammalian reproductive tract. It is important for follicle growth, oocyte maturation, and fertility (Jamnongjit & Hammes, 2006). Estradiol (E2) is primarily synthesized within the follicles of the ovaries via the process of ovarian steroidogenesis (Nakamura et al., 2009). E2 is essential for several functions such as: maturation of the oocyte, development of female secondary sexual characteristics, fertility, and health (Nakamura et al., 2009). Although ovarian steroidogenesis is well described in the literature and has been researched for many years, much is unknown about this elaborate process.

Sirtuins (SIRTs) are enzymes that can be found in almost all domains of life and they act as mediators in several cellular pathways (Chang & Guarente, 2014). SIRTs are defined as class III histone deacetylases dependent of NAD+. They are a family that consists of seven members, SIRT 1 - 7 (Michan & Sinclair, 2007). SIRT1, SIRT3, and SIRT5 have been reported to act in the ovaries by mediating several biological processes, including ovarian steroidogenesis and some diseases, such as. polycystic ovarian syndrome (Dong et al., 2016; Tan et al., 2015) In addition, SIRTs interact with various hormones in the mammalian female (Sirotkin et al., 2020; Tao et al., 2015; Zhang et al., 2016). Therefore, a comprehensive study investigating the role of SIRTs on granulosa cells (GC) is important for the understanding of molecular regulation of ovarian steroidogenesis. We hypothesize that factors that regulate ovarian steroidogenesis influence sirtuins gene expression and activity in GC.

In this study, we used bovine GC to investigate the association between steroidogenesis and sirtuins expression and activity in a mammalian mono-ovulatory species. Different hormones involved in the processes of ovarian steroidogenesis, cellular proliferation, and cellular differentiation were used to treat bovine GC in vitro.

Material and Methods

Reagents and hormones

The reagents used to perform cell culture were the following: DMEM/Ham's F-12 (Genesee Scientific, San Diego, CA); gentamicin, sodium bicarbonate, DNase I, collagenase, and trypan blue (MilliporeSigma, St. Louis, MO); fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO); testosterone (Steraloids, Newport, RI); purified ovine follicle-stimulating hormone (FSH; Scripps lab, San Diego, CA); insulin-like growth factor I (IGF-1), fibroblast growth factor 2 (FGF-2), fibroblast growth factor 9 (R&D Systems, Minneapolis, MN).

Granulosa cell culture

Bovine ovaries were collected at an USDA-inspected commercial slaughterhouse. Following collection, ovaries were washed once with saline 0.9%, once with 0.9% saline plus 70% isopropanol, and kept on ice with antibiotic saline (0.9% saline solution with 1% streptomycin; PH: 7.4) to be transported to laboratory. Follicular fluid was aspirated from small antral follicles (1-5 mm on surface diameter) using needle (20G) and 3 mL syringe (BD, Franklin Lakes, NJ). The aspirated follicular fluid was centrifuged at 200 x g for 4 min at 4°C and supernatant was discarded. Cell pellets then received serum-free medium (1:1 DMEM/Ham's F12 supplemented with 0.12 mM gentamycin and 23.8 mM sodium bicarbonate) and another centrifugation at 200 x g for 4 min at 4°C was performed. Supernatant was once again discarded, and cell pellets were then resuspended in serum-free medium supplemented with 1.25 mg/ml collagenase and 0.5 mg/ml DNAse to avoid clumping. After resuspension, GC viability was defined using trypan blue exclusion method and averaged 45% for the experiments. On average, $2.5X10^5$ viable cells were seeded per well on 24-well plates (Corning, Corning, NY) in 1 mL of DMEM/Ham's F12 medium containing 10% FBS at 38.5 °C in a 5% CO2 controlled atmosphere. Prior to treatment, cells were allowed to grow from 48 h to 60 h, depending on the experiment, and medium was replaced every 24 h. Cells were treated when reaching an average cellular confluence of approximately 75%. Before treatments, cells were washed twice with 0.5 mL of serum-free medium and treatments were applied in serum-free medium for RNA extraction or for collection of cell protein. Each treatment was applied to two wells in all experiments.

Experimental design

Experiments were conducted to determine the influence of hormones known to regulate ovarian steroidogenesis on sirtuins gene expression (Experiment 1) and activity (Experiment 2) in GC. Cells were treated with FSH (30ng/mL), IGF1 (30ng/mL), FGF-2 (30 ng/ml), FGF-9 (30ng/ml) and their combinations in both experiments. The doses of FSH, IGF-1, FGF2, and FGF9 were selected based on previous studies (Schreiber & Spicer, 2012; Spicer et al., 2002; Spicer et al., 1993). These treatments were selected because FSH alone is known to stimulate E2 production of GC and this stimulus is further increased in the presence of IGF-1 (Spicer et al., 2002) whereas FGF9 decreases FSH and IGF-1-stimulated E2 production (N. B. Schreiber & Spicer, 2012) and FGF2 decreases IGF-1 receptor abundance and FSH-induced E2 production (Spicer et al., 1993). All cells received 500 ng/mL of testosterone as a substrate for E2 production.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qPCR)

Total RNA from GC (n = 6 pools) was isolated using miRNeasy mini kit (Qiagen, Hilden Germany) following to the manufacturer's protocol. Medium was aspirated from the wells and 500 µL of Qiazol lysis buffer was added to the cells in each well. Samples from each treatment were collected from two wells and placed in a 1.7 mL microcentrifuge tube and stored at -80^{o}C for RNA extraction. Following RNA extraction, RNA concentration and integrity were determined by NanoDrop One spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Samples were stored at -80^{o}C for qPCR analysis.

For analysis of relative gene expression, an average of 600ng of total RNA was used to generate cDNA by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Target genes mRNA relative abundance was measured by qPCR with a CFX Connect Real-Time PCR Detection System (BioRad, Hercules, CA, USA) using a SYBR select Master Mix (Applied Biosystems, Waltham, MA). Primers for SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 were designed by Primer Express software v3.0.1 (Applied Biosystems, Waltham, MA), following the National Center for Biotechnology Information (NCBI) Reference Sequence database (https://www.ncbi.nlm.nih.gov). Primers sequences, and annealing temperature are described in Table 3. The qPCR temperature protocol was: 95^{o}C for 10 min (1 cycle), 95°C for 15 seconds followed by 65°C for 1 min (40 cycles) for SIRT2, SIRT3, SIRT6, SIRT7, and 60°C for 1 min (40 cycles) for SIRT1, SIRT4, SIRT5. Normalization of expression of

target genes was done by 18S ribosomal RNA (18S) expression levels. The relative abundance of mRNA was calculated by the 2-ΔΔCt equation and expressed as fold change compared to the negative control group (Livak & Schmittgen, 2001).

Table 3 – Primers References

Primers	**NCBI Reference**	**Optimal temperature (Annealing, extension, and read fluorescence)**	**Primer seq by Primer Express software v3.0.1 (Applied Biosystems, Waltham, MA),**
SIRT 1	NM_001192980.3	60 °C	**FWD**: CAACAGCATCTTGCCTGATTTG **REV**: TTTCATGATAGCAAGCGGTTCA
SIRT 2	NM_001113531.1	65 °C	**FWD:** CGCAGGGTCATCTGTTTGGT **REV**: GGCATAGAGGCCCGTGTTT
SIRT 3	XM_005200933.4	65 °C	**FWD**: TGGGCTGACGTTGCTGTTT **REV**: TGATTCAAGAGATTCGCCATCA
SIRT 4	NM_001075785.1	60 °C	**FWD**: CAGGTCAGAAAAGGTGGGACTTT **REV**: GTACAAAATCCCCATGCTGGAT
SIRT 5	GQ166651.1	60 °C	**FWD**: TGGCCGAATTCAACATGGA **REV**: ACGGCCCCTGGAAATGA
SIRT 6	NM_001098084.1	65 °C	**FWD**: TCGCCTGGTCATCGTCAA **REV**: CATCAACATAACCGTGGATTCG
SIRT 7	NM_001075217.1	65 °C	**FWD**: GCTCCACGGGAACATGTACA **REV**: CATCAAACACCCGCACATATTC

FWD: forward primer (anti-sense strand); **REV**: reverse primer (sense strand).

Sirtuins activity

To measure sirtuins activity, nuclear and cytoplasmic extracts from GCs (n=3 pools) after treatments were isolated using the Epiquick nuclear extraction kit (Epigentek, Farmingdale, NY, USA) following the manufacturer's instructions. The Epigenase Universal SIRT Activity/Inhibition Assay Kit (Epigentek, Farmingdale, NY, USA) was used to evaluate sirtuins. In this assay, we utilized 2 μg of nuclear fraction for SIRT1, SIRT6, SIRT7 activity and 2 μg of cytoplasmic fraction for SIRT2, SIRT4 and SIRT5 activity in each assay kit following the manufacturer's instructions. Nuclear and cytoplasmic SIRTs activity is calculated as ng/min/mg.

Statistics

Data were analyzed via ANOVA with GLM procedures of SAS for Windows (version 9.4, SAS Institute Inc., Cary, NC). Values were submitted to Shapiro-Wilk test of normality and were transformed to natural log (x+1) to ensure homogeneity of variance when necessary. Data were presented as means of experimental groups ± SEM. Mean differences were assessed using Fisher's protected least significant differences test. Differences among treatments were considered significant at $p < 0.05$. Outliers were detected through Dixon's test and were excluded from analyses.

Results

Quantitative real-time reverse transcription polymerase chain reaction (qPCR)

Various hormones altered SIRTs mRNA relative expression in GC. *SIRT1* mRNA abundance was increased 2-fold ($p<0.05$) by FGF2 alone in comparison to negative control (Figure 1). *SIRT2* mRNA abundance was increased by FSH+IGF1+FGF9 in comparison to all treatments ($p<0.05$), except for FSH+IGF1+IGF2 ($p=0.0664$) and control group and was increased by FSH+IGF1+FGF2 ($p<0.05$) in comparison to FSH+IGF-1 (Figure 2). *SIRT3* mRNA relative abundance was increased by FSH+IGF1+FGF9 in comparison to every single treatment ($p<0.05$) and was also increased by FSH+IGF1+FGF2 in comparison to FGF2 alone ($p<0.05$) and FSH alone ($p <0.0001$) (Figure 3). *SIRT4* mRNA abundance was increased by FSH+IGF1+FGF9 in comparison to all treatments ($p<0.05$), except in comparison to FGF9 alone and was also increased by FSH alone ($p<0.05$) and FGF9 alone ($p<0.05$) in comparison to negative control (Figure 4). *SIRT5* mRNA relative abundance was increased by FSH+IGF1+FGF9 in comparison to negative control ($p<0.05$) and IGF1 alone ($p<0.05$) (Figure 5). *SIRT6* mRNA relative abundance was increased by FSH+IGF1+FGF9 in comparison to all treatments ($p<0.05$) and was also increased by FSH+IGF1+FGF2 in comparison to FSH+IGF1 ($p<0.05$) and FGF9 ($p<0.05$) treatments (Figure 6). *SIRT7* mRNA relative abundance was increased by FSH+IGF1+FGF9 in comparison to all treatments ($p<0.05$), except to negative control and FSH+IGF1+FGF2 and was also increased by FSH+IGF1+FGF2 in comparison to FSH alone and tended to be increased in comparison to FGF2 alone (Figure 7).

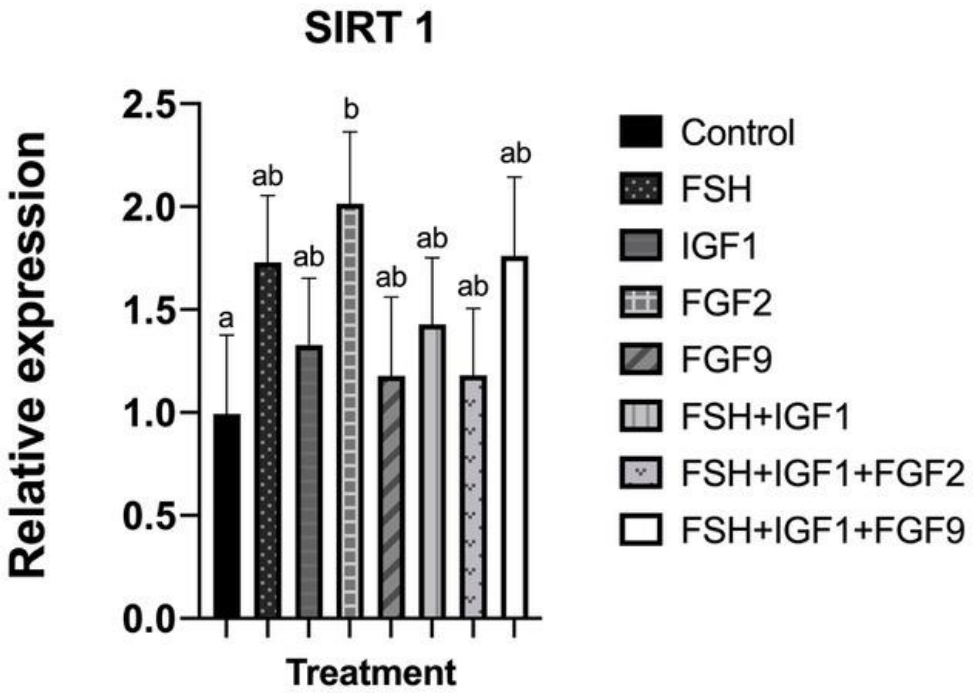

Figure 1 – Sirtuin 1 relative gene expression under different hormone treatments. Sirtuin 1 relative gene expression under different hormone treatments in bovine granulosa cells (GC). Means ± SEM without a common letter differ ($p < 0.05$)

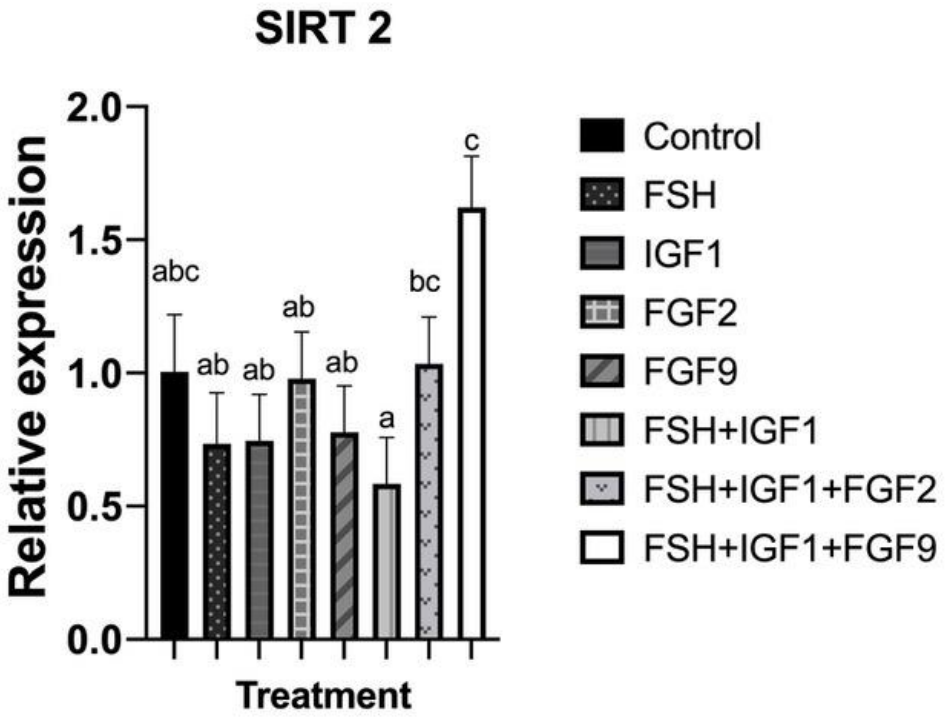

Figure 2 – Sirtuin 2 relative gene expression under different hormone treatments. Sirtuin 2 relative gene expression under different hormone treatments in bovine granulosa cells (GC). Means ± SEM without a common letter differ ($p < 0.05$)

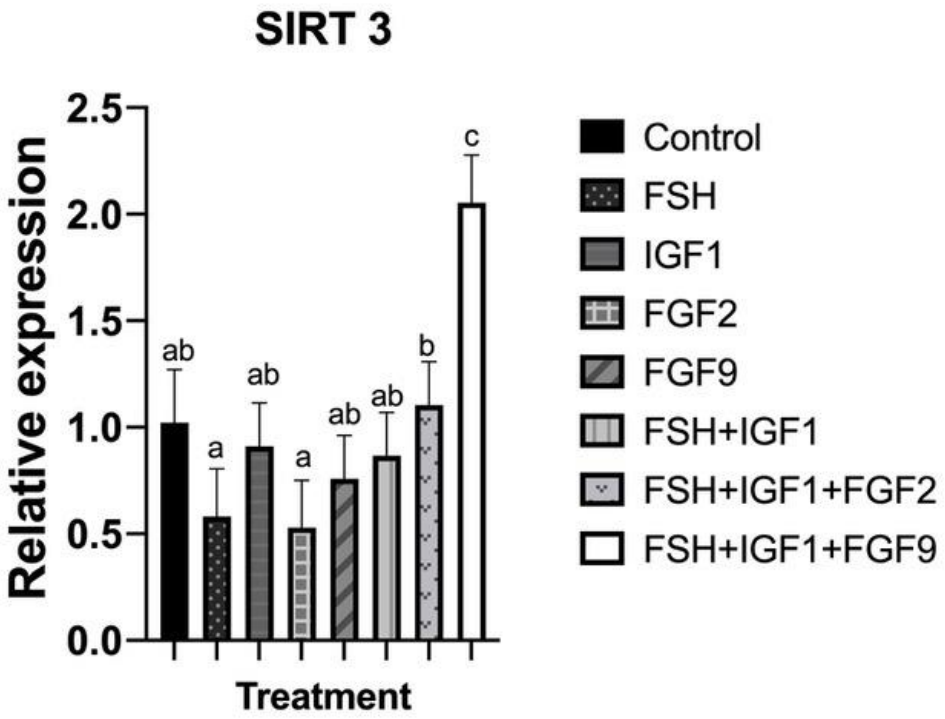

Figure 3 – Sirtuin 3 relative gene expression under different hormone treatments. Sirtuin 3 relative gene expression under different hormone treatments in bovine granulosa cells (GC). Means ± SEM without a common letter differ ($p < 0.05$)

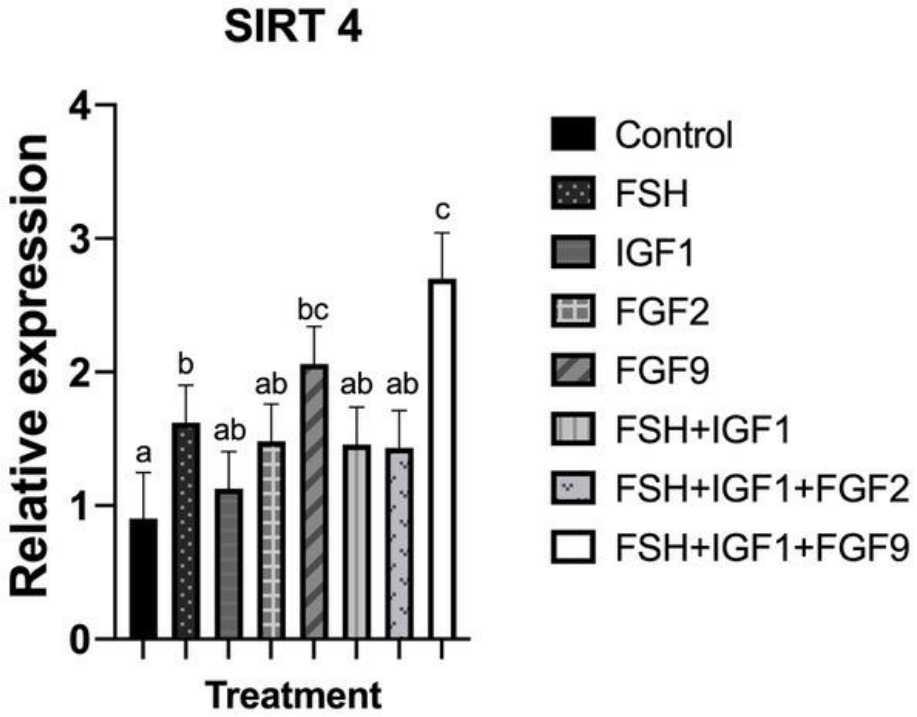

Figure 4 – Sirtuin 4 relative gene expression under different hormone treatments. Sirtuin 4 relative gene expression under different hormone treatments in bovine granulosa cells (GC). Means ± SEM without a common letter differ ($p < 0.05$)

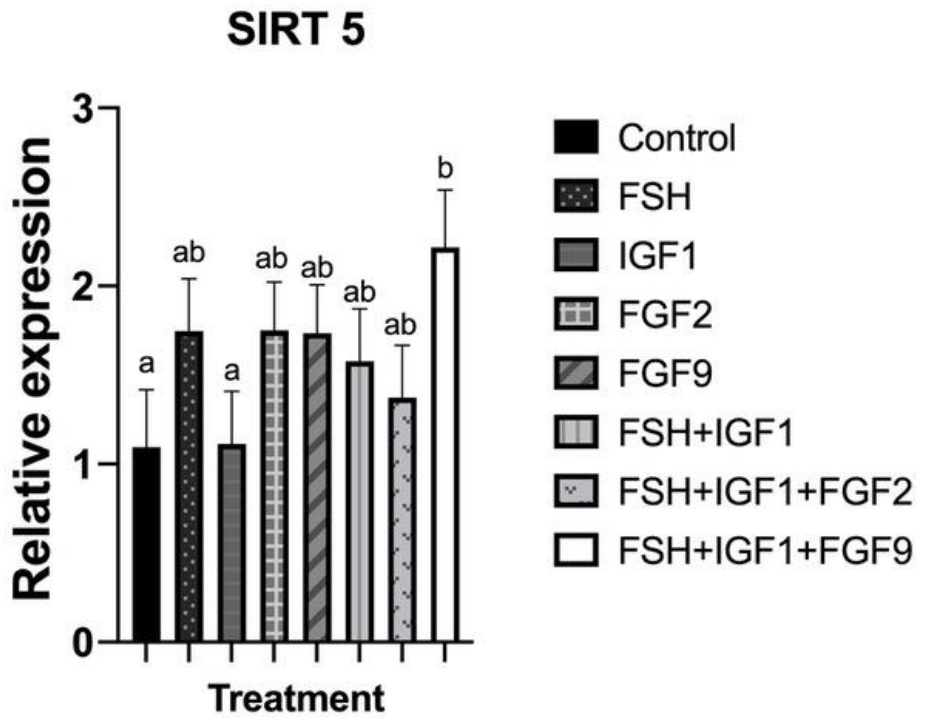

Figure 5 – Sirtuin 5 relative gene expression under different hormone treatments. Sirtuin 5 relative gene expression under different hormone treatments in bovine granulosa cells (GC). Means ± SEM without a common letter differ ($p < 0.05$)

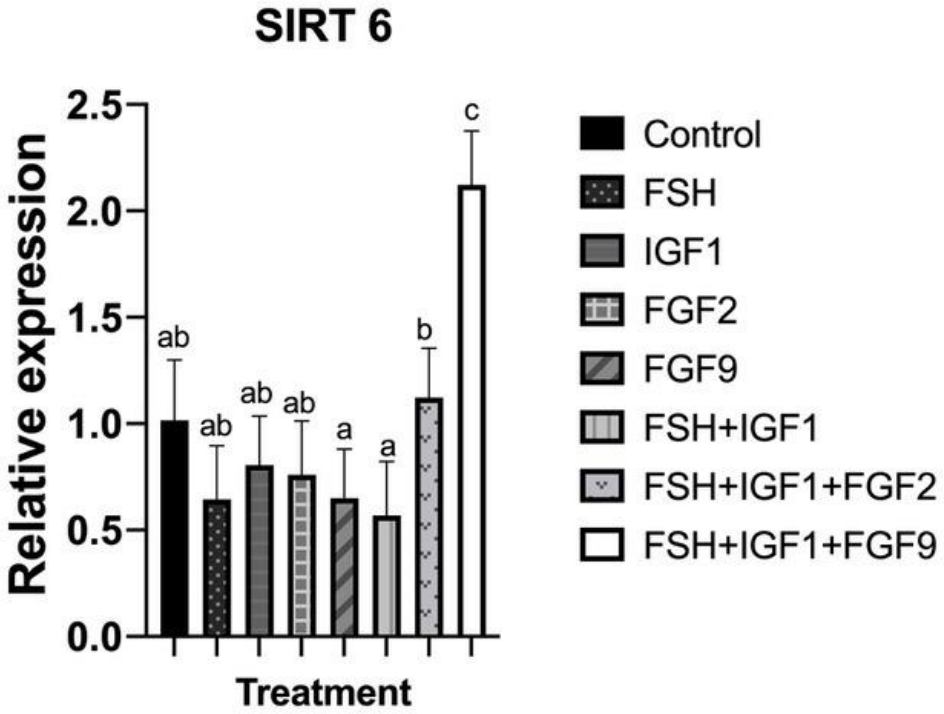

Figure 6 – Sirtuin 6 relative gene expression under different hormone treatments. Sirtuin 6 relative gene expression under different hormone treatments in bovine granulosa cells (GC). Means ± SEM without a common letter differ ($p < 0.05$)

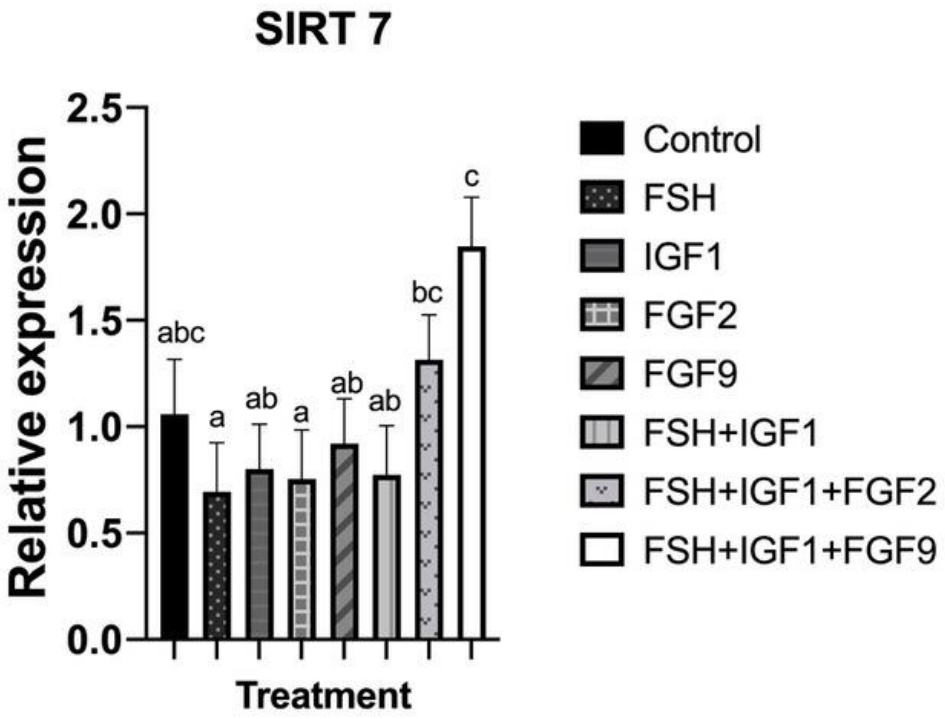

Figure 7 – Sirtuin 7 relative gene expression under different hormone treatments. Sirtuin 7 relative gene expression under different hormone treatments in bovine granulosa cells (GC). Means ± SEM without a common letter differ ($p < 0.05$)

Sirtuins Activity

In term of nuclear SIRTs (SIRTs 1, 6, and 7) activity, FGF2 alone increased ($p<0.05$) nuclear SIRTs activity in comparison to FSH alone (2.6-fold), IGF1 alone (2-fold), FSH+IGF1 (2.44-fold), and FGF9 alone (1.75-fold). Also, there was a tendency ($p = 0.06$) for FSH+IGF1+FGF2 to be greater (1.90-fold) than FSH alone (Figure 8).

In terms of cytoplasmic SIRTs (SIRTs 2, 4, and 5) activity, FSH+IGF1+IGF2 increased cytoplasmic SIRTs activity in comparison to all treatments ($p< 0.05$) (Figure 9).

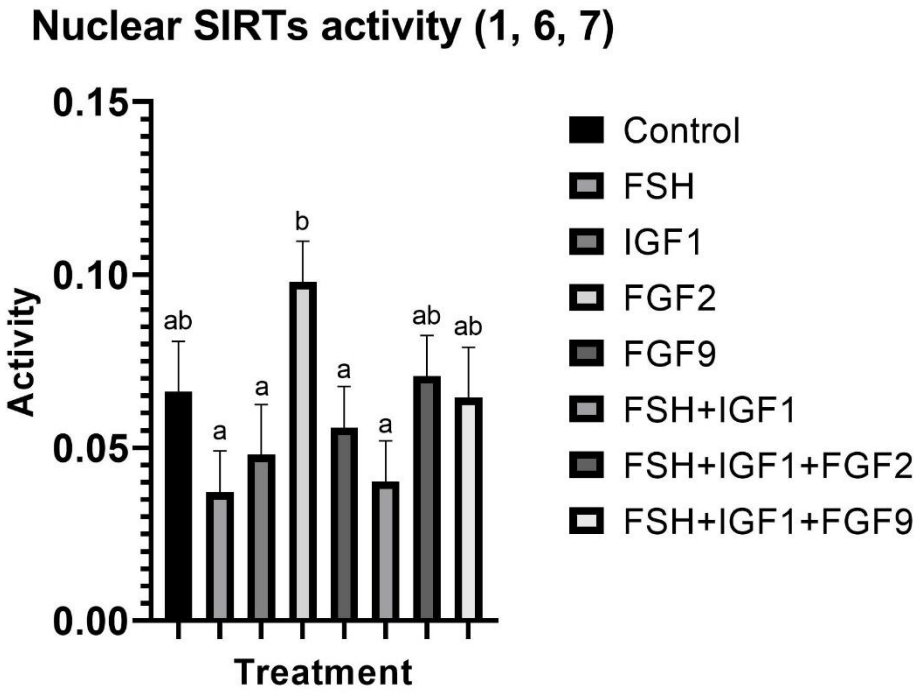

Figure 8 – Nuclear Sirtuins activity. Nuclear sirtuins activity in response under different hormonal treatments in bovine granulosa cells Means ± SEM without a common letter differ ($p<0.05$)

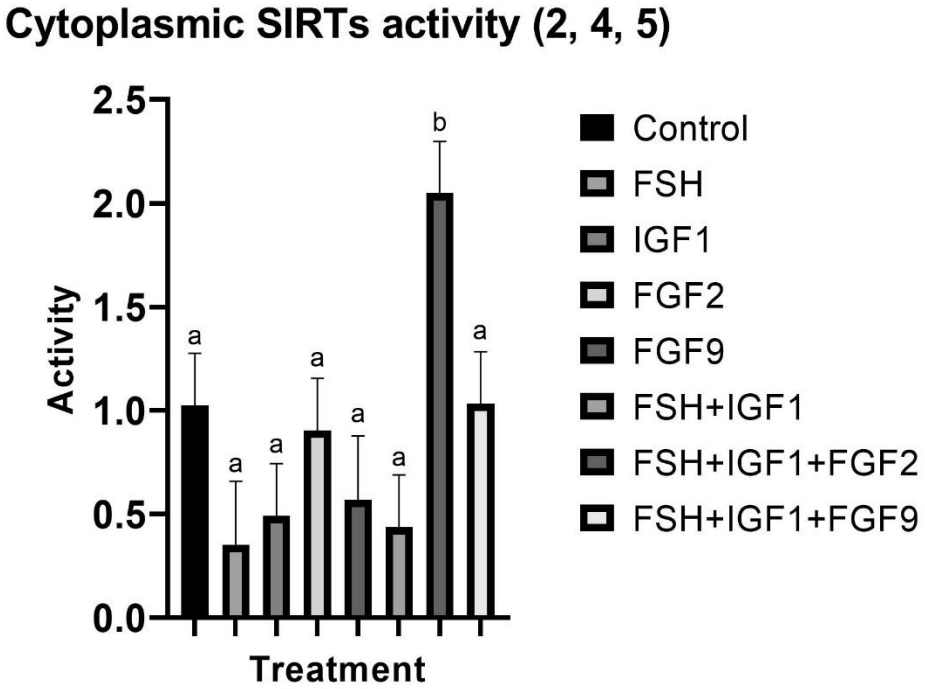

Figure 9 – Cytoplasmic Sirtuins activity. Cytoplasmic sirtuins activity in response under different hormonal treatments in bovine granulosa cells Means ± SEM without a common letter differ ($p <0.05$)

Discussion

SIRTs are involved in a variety of cellular functions such as: gene silencing, control of the cell cycle, apoptosis and energy homeostasis (Yamamoto et al., 2007). All seven SIRTs are expressed in the mammalian ovary (Aljada, 2015; Kong et al., 2020; Morita et al., 2012; L. Pacella-Ince et al., 2014a; Pacella-Ince et al., 2014b; Xu et al., 2019; Zhao et al., 2014). Although studies have reported an association between sirtuins in GC and steroidogenesis, it is important to understand how SIRTs expression and activity in GC are regulated.

The current study evaluated the hormonal regulation of SIRTs mRNA expression in bovine GC. Among the hormones tested, FGF2 and FGF9 were the most impactful to SIRTs mRNA abundance. Specifically, FGF2 alone increased SIRT1 mRNA relative abundance under basal culture conditions whereas, in the presence of FSH plus IGF-1, both FGF2 and FGF9 increased SIRT2 and SIRT6 mRNA abundance and FGF9, but not FGF2, increased SIRT3 and SIRT4 mRNA abundance. The combination of FSH, IGF-1, and FGF2 increased SIRT3 mRNA relative abundance in comparison to FGF2 alone. SIRT5 was the only SIRT not affected by FGF2 and FGF9 and only SIRT4 mRNA relative abundance was increased by FSH under basal culture conditions. Taken together, these results suggest a stimulatory effect of FGF2 and FGF9 on various SIRTS mRNA relative abundance. The fact that FGF2 and FGF9 are considered anti-differentiation factors of granulosa cells and inhibit FSH-induced steroidogenesis (N. B. Schreiber & Spicer, 2012; Vernon & Spicer, 1994) suggest that SIRTs expression are important to prevent GC steroidogenesis and differentiation. Indeed, in cattle, activation of GC SIRT1 expression decreased E2 production (Tan et al., 2015) whereas SIRT3 knockdown in human GC from preovulatory follicles resulted in decreased mRNA expression of CYP19A1, STAR, and CYP11A1 and progesterone secretion (Fu et al., 2017).

Nuclear SIRTs activity (SIRTs 1, 6, and 7) was increased by FGF2 alone in comparison to various treatments, behaving similarly to SIRT1 mRNA relative abundance, which was increased in response to FGF2 alone under basal culture conditions. FGF2 in combination with FSH and IGF1 also increased transcripts of SIRT6 in comparison to FSH+IGF1. Because FGF2 alone is known to stimulate bovine GC proliferation, (Gospodarowicz et al., 1977) it might be the case that nuclear SIRTs activity is especially important to regulate cell proliferation.

The combination of FGF2 with FSH plus IGF1 was the most impactful treatment to cytoplasmic SIRTs (SIRTs 2, 4, and 5) activity. This combination also increased SIRT2 transcripts in comparison to FSH plus IGF-1. Because FGF2 suppresses FSH-induced E2 production and decreases IGF-1 receptors abundance in bovine GC (Vernon & Spicer, 1994), cytoplasmic SIRTs activity might be negatively correlated with follicular dominance in cattle. Future studies should be conducted to confirm whether FGFs regulate steroidogenesis and GC differentiation via regulation of SIRTs activity in cattle.

In conclusion, the findings of the current study indicate that FGFs 2 and 9 stimulate SIRTs activity and mRNA relative abundance in bovine GC. These findings suggest that FGFs may regulate folliculogenesis and ovarian steroidogenesis through SIRTs activity and gene expression in GC of cattle.

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