Stressors modulating granulosa cell functionality, ovulation and oocyte competence in bovines

Kumulative Dissertation

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Rostock

vorgelegt von

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geb. am 11.08.1987 in Kanaid (Himachal Pradesh)

aus Indien

Rostock, May 2020

https://doi.org/10.18453/rosdok_id00002985

Day of Submission: 15.05.2020

Day of Doctoral Defense: 08.01.2021

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Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
ALA	α-linolenic acid
$ApoE^{-/-}$	Apolipoprotein E-deficient
ArKO	Aromatase gene knockout
BAECs	Bovine aortic endothelial cells
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BHBA	β-hydroxybutyric acid
BMI	Body mass index
BSA	Bovine serum albumin
C1QTNF3	Complement C1q tumor necrosis factor-related protein 3
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
CCND2	Cyclin D2
Cd11b	Cluster of differentiation molecule 11b
CD36	Cluster of differentiation 36
CDKN	Cyclin dependent kinase inhibitor
ChIP	Chromatin precipitation
CHO	Chinese hamster ovary
CL	Corpus luteum
CLA	Conjugated linoleic acid
CO_2	Carbon dioxide
COC	Cumulus -oocyte complex
COFs	Cystic ovarian follicles
CTRL	Control
CYP19A1	Cytochrome P450, family 19, subfamily A member1
DMRs	Differentially methylated regions
DNA	Deoxyribonucleic acid
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
E2	17β-Estradiol
EFAs	Essential fatty acids

EMT ..... Epithelial-mesenchyme transition
ER ..... Endoplasmic reticulum
ERE ..... Estrogen response element
ERK1/2 ..... Extracellular signal-regulated kinases ½
ESR1 ..... Estrogen receptor 1
$FABP_{pm}$ ..... Plasma membrane associated fatty acid binding protein
FATP 1-6 ..... Fatty acid transport proteins 1-6
FF ..... Follicular fluid
FFAs ..... Free fatty acids
FOXL2 ..... Forkhead box protein L2
FSH ..... Follicle stimulating hormone
FSHR ..... Follicle stimulating hormone receptor
FSH-β ..... Follicle stimulating hormone-β subunit
G1-S Gap1-synthesis
Gata 4 ..... GATA binding protein 4
GCs ..... Granulosa cells
Gdf 9 ..... Growth differentiation factor 9
GLUT 1 ..... Glucose transporter 1
GLUT 3 ..... Glucose transporter 3
GLUT 4 ..... Glucose transporter 4
GnRH ..... Gonadotropin-releasing hormone
GPCRs ..... G protein-coupled receptors
HBA1 ..... Hemoglobin alpha 1
hCG ..... Human chorionic gonadotropin
HIF1α ..... Hypoxia inducible factor-1alpha
HIFs ..... Hypoxia-inducible factors
HIST1H2BN ..... Histone H2B type 1-N
HRE ..... Hypoxia response element
HSD3B1 ..... Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
ICAM1 ..... Intercellular adhesion molecule 1
IGF-1 ..... Insulin-like growth factor-1
IGF-1R ..... Insulin-like growth factor 1 (IGF-1) receptor
IL6 ..... Interleukin 6

IL8 ..... Interleukin 8
IPA ..... Ingenuity pathway analysis
IVC ..... *in vitro* culture
IVF ..... *in vitro* fertilization
IVM ..... *in vitro* maturation
IVP ..... *in vitro* embryo production
JNK ..... c-Jun N-terminal kinases
LA ..... Linoleic acid
LC-MS ..... Liquid chromatography-mass spectrophotometry
LHCGR ..... Luteinizing hormone/choriogonadotropin receptor
MAPK ..... Mitogen-activated protein kinases
miRNAs ..... MicroRNAs
MPMs ..... Mouse peritoneal macrophages
mRNA ..... messenger ribonucleic acid
MUFAs ..... Mono-unsaturated fatty acids
NADPH ..... Nicotinamide adenine dinucleotide phosphate
NEB ..... Negative energy balance
NEFAs ..... Non-esterified fatty acids
NF-κB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells
O_2 ..... Oxygen
OA ..... Oleic acid
P4 ..... Progesterone
P450scc ..... Cytochrome P450 side-chain cleavage enzyme
PA ..... Palmitic acid
PCNA ..... Proliferating cell nuclear antigen
PCOS ..... Polycystic ovarian syndrome
PGFM 13, 14-dihydro-15-keto-prostaglandin F2α
pH ..... Potential of hydrogen
PI3K ..... Phosphoinositide 3-kinase
PKA ..... Protein kinase A
PKB ..... Protein kinase B
PMSG ..... Pregnant mare serum gonadotropin
PPARs ..... Peroxisomal proliferator-activated receptors

PPI.....Protein-protein interaction
PTGS2.....Prostaglandin-endoperoxide synthase 2
PUFAs.....Polyunsaturated fatty acids
RIF .....Rumen inert fat
ROS.....Reactive oxygen species
RT-qPCR .....Real time-quantitative polymerase chain reaction
SA .....Stearic acid
SCD1.....Stearoyl-CoA desaturase 1
SF-1.....Splicing factor-1
SFAs.....Saturated fatty acids
siRNA .....Small interfering RNA
SOX 9 .....SRY (sex determining region Y)-box 9
SSO .....Sulfosuccinimidyl oleate
StAR.....Steroidogenic acute regulatory protein
TCN .....Total cell number
TNF .....Tumor necrosis factor
TNFAIP3 .....TNF alpha-induced protein 3
TNFAIP8 .....TNF alpha-induced protein 8
TUNEL .....Terminal deoxynucleotidyl transferase dUTP nick end labeling
TXNIP.....Thioredoxin interacting protein
UFAs.....Unsaturated fatty acids
VCAM1 .....Vascular cell adhesion protein 1
VNN 1.....Vanin 1
VNN2.....Vanin 2

Standard units and symbols:

%.....Percentage
°C .....degree Celsius
µM.....micromolar
mM.....millimolar
µm.....micrometer
µmol/l.....micromoles per liter
kDa.....kilo Daltons
kg/m^2……kilogram per meter square
mg/dl……milligrams per deciliter
mm ……..millimeter
mM……...millimolar
ng……..nanogram
ng/ml……nanogram per milliliter
pg ……....picogram

1. Introduction

The ovarian follicle provides an ideal environment for oocyte maturation and ovulation by providing the necessary nutrients and regulatory agents such as steroid hormones, growth factors, and cytokines. However, beside these timely endocrine stimulations follicular development is extremely sensitive towards the health and energy status of the animal. A compromised health status due to various physical and metabolic constrains can affect the fertility and productivity. Typical stressors experienced by cattle include environmental, nutritional, physiological and psychological factors. Most relevant stressors affecting reproductive processes in dairy cattle are heat stress and imbalanced metabolism arising due to negative energy balance (NEB) during the transition period. These stress conditions affect uterine health, ovarian function, oocyte quality, and the capacity for conception of the animal. Stressors that affect the ovarian function, particularly steroidogenesis in granulosa cells (GCs), oocyte development and ovulation in bovine are the prime topics of the present dissertation. As a background to aid understanding of alterations elicited by these stressors, normal ovarian folliculogenesis and steroidogenesis are briefly described.

1.1 Ovarian follicular development

1.1.1 Folliculogenesis

Mammalian folliculogenesis and oogenesis occur concurrently in a coherent manner inside the ovarian follicles. The ovarian follicle is a specialized structure consisting of a female germ cell, the "*oocyte*", surrounded by specialized somatic cells. The oocyte develops and reaches ovulatory maturity inside the follicle. "*Folliculogenesis"* is the maturation of the ovarian follicle, which begins with the accumulation of primordial follicles within the ovary during fetal life in both human and bovine. Primordial follicles consist of a centrally located immature oocyte surrounded by a single layer of flattened pre-granulosa cells. Activation of primordial follicles leads to the transition into primary follicles with cuboidal shaped GCs. This is accomplished by proliferation and differentiation of GCs. Once the primary follicle starts progressive growth, numerous factors such as insulin-like growth factor 1 (IGF-1), gonadotropins and steroid hormones contribute to sustain the growth of the follicle into the fully

mature Graafian follicle ready for ovulation. Primary follicles express receptors for follicle stimulating hormone (FSH) and subsequently the cytoplasmic volume of the oocyte is expanded and a glycoprotein polymer capsule, the "*zona pellucida*", is formed between the growing oocyte and GCs. Stromal theca cells are assembled by signals secreted by the oocyte. Theca cells surround the follicle outermost layer, the basal lamina and undergo cytodifferentiation to form the theca externa and theca interna. A network of vascular capillaries forms between the two theca layers and begins circulating blood to and from the follicle (Aerts and Bols, 2010b).

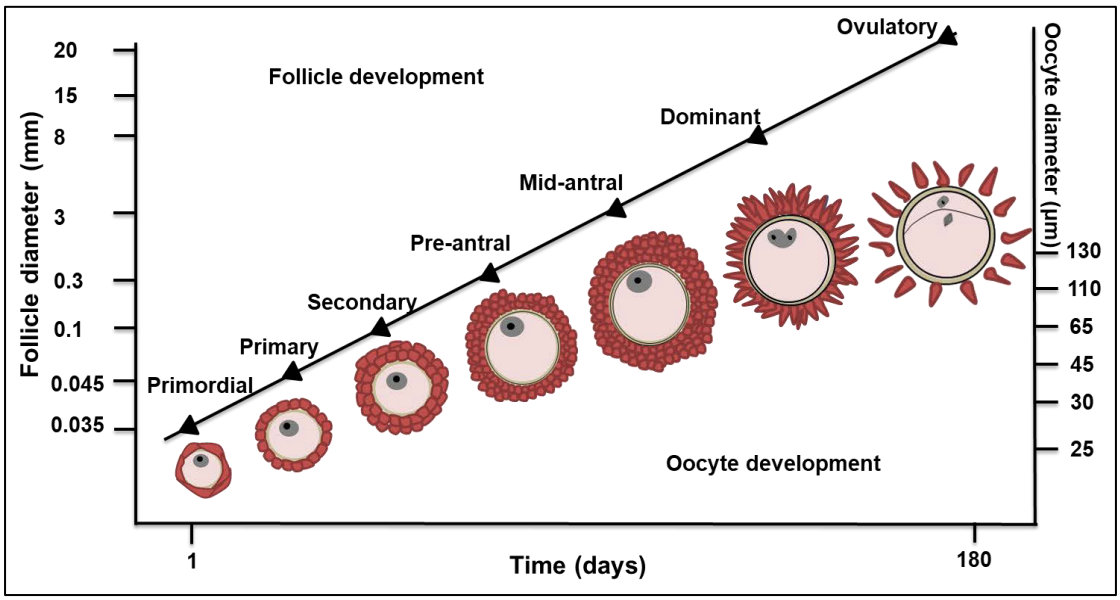

Figure 1. Correlation between follicle and oocyte development in cattle. The oocyte growth correlates with follicle diameter, oocyte diameter, duration and stages of folliculogenesis from primordial till preovulatory phase (Fair, 2003). (Lussier *et al.,* 1987) (Hulshof *et al.,* 1994).

A fully formed secondary follicle is characterized by a grown oocyte surrounded by a zona pellucida, 3-4 layers of GCs, a basal lamina, a theca interna, a capillary net and a theca externa layer. It is during the secondary follicle stage that the majority of activated follicles evolve to the antral stage characterized by the presence of an antral cavity often referred to as "*antrum*". Development of meiotic competence commences with the transition of pre-antral into antral follicle. Pre-antral follicles are meiotically incompetent and are unable to develop beyond the diplotene stage of meiosis I. Bovine oocytes become meiotically competent at a size of 110 to 115 µm and the developmental competence is acquired at 120 µm. Bovine *in vitro* matured oocytes can develop to the blastocyst stage until the oocyte expansion reaches a size

of 135 µm (Armstrong and Webb, 1997) (Figure 1). The development of a secondary follicle into a preovulatory follicle is gonadotropin dependent as GCs of the follicle are stimulated by FSH. Synergistically with FSH, IGF-1 induces proliferation and steroidogenesis (discussed later) in GCs thus promoting the formation of a follicular antrum. In response to FSH and IGF-1, recruited antral follicles also secrete the female sex steroid, 17β-estradiol (E2) which further induces the release of another gonadotropin, luteinizing hormone (LH) from anterior pituitary. The antral follicle that reaches the appropriate growth at a precise threshold of the ovarian cycle becomes the dominant follicle during each estrus cycle. The preovulatory LH surge further triggers the release of a mature oocyte from the fully developed dominant follicle competent for fertilization. The remaining somatic cells (i.e. theca cells and GCs) further transdifferentiate into the corpus luteum (CL) after ovulation. The CL secretes large amounts of progesterone (P4), a steroid hormone essential for establishing and maintaining pregnancy in females (Aerts and Bols, 2010a).

1.1.2 Follicular fluid (FF)

A nutrient enriched preovulatory follicular environment is indispensable for the residing cumulus-oocyte complex (COC) to complete its cytoplasmic and nuclear maturation. Large mammalian species such as human, bovine, equine, porcine and ovine have large follicles with relatively higher amounts of FF as compared to small mammalian species such as rat and mice (Rodgers *et al.,* 2001). The formation of FF begins with the vascularization of the theca layers. Fluid from theca capillaries passes through the endothelium and the sub-endothelial basal lamina into the interstitium and then through the follicular basal lamina and the membrana granulosa finally reaching the antrum. Thus, any changes in permeability of the thecal capillaries might lead to edema in the thecal tissue (Cavender and Murdoch, 1988). The blood follicle barrier is selectively permeable as it allows the passage of only low molecular weight proteins of up to 500 kDa (Hess *et al.,* 1998) (Shalgi *et al.,* 1973). The components of the FF surrounding the COC at the time of *in vivo* cumulus expansion are hyaluronan, versican, and inter-α trypsin inhibitor (Clarke *et al.,* 2006) (McArthur *et al.,* 2000) (Rugg *et al.,* 2005). The composition of the FF keeps changing throughout the estrus cycle in cows (Orsi *et al.,* 2005). However, under specific physiological conditions biochemical composition of FF might impose negative effects on the overall ovarian function.

A higher concentration of non-esterified fatty acids (NEFAs), β-hydroxy butyrate, and urea have been found in metabolic stressed and emaciated ewes (Farman *et al.,* 2015). Correlation between serum and FF metabolites was reported in postpartum cows. Accordingly, changes in serum metabolite concentrations are reflected in the FF and may impair fertility during lactation (Alves *et al.,* 2014). Apart from metabolites, several studies in transgenic knockout mice have also demonstrated the role of various regulatory molecules that are involved in FF formation. Mice null mutants for growth differentiation factor 9 (Gdf9) (Dong *et al.,* 1996), follicle-stimulating hormone-β subunit (FSH-β) (Kumar *et al.,* 1997), follicle stimulating hormone receptor (FSHR) (Abel *et al.,* 2000) and IGF-1 (Zhou *et al.,* 1997) were found to have defects during the preantral or early antral follicle development. Likewise, disruption of estrogen receptor 1 (*ESR1*) gene (Lubahn *et al.,* 1993) and aromatase gene (Britt *et al.,* 2000) in mice developed large cystic hemorrhagic follicles, which clearly indicates that these regulatory molecules are essential for FF accumulation. Thus, it is very clear that the presence of nutritional and metabolic stressors above physiological levels in FF could negatively affect fertility through a reduction of the oocyte developmental competence as well as an alteration in the expression of important regulatory molecules.

1.1.3 GCs and steroidogenesis

GCs are supposed to originate from the ovarian surface epithelium. The progenitor cells called pregranulosa cells surround the primordial oocyte. About 3-5 pregranulosa cells give rise to a set of fully functional GCs in a mature follicle. GCs rest on a basal lamina that separates them from the vascularized theca interna. As a result there is a relative blood-follicle barrier that restricts access of high molecular weight substances, such as low-density lipoproteins into the GCs compartment. An interesting aspect of GCs is their functional heterogeneity depending on the location within the follicle itself. Mural GCs, antral GCs and cumulus GCs each have functions that depend on their proximity to the oocyte and theca cells. Mural GCs lines the follicular wall around the antrum, antral GCs reside in follicle antrum and cumulus GCs lie in close contact to the oocyte. The murals GCs have high steroidogenic activity and express high LH receptors as compare to cumulus GCs. Cumulus GCs possess high proliferation capacity (Li *et al.,* 2000) (Eppig *et al.,* 1997).

GCs play an indispensable role in maintaing the normal ovarian function as the key steroid hormones, E2 and P4 are secreted by GCs. In general, synthesis of these steroid hormones is referred to as "*steroidogenesis*". Ovarian follicular steroidogenesis requires a cooperative action between theca cells and antral GCs (Figure 2). Steroidogenesis commences with internalization of cholesterol in form of low-density-lipoprotein into the theca cells *via* receptor mediated endocytosis. Once inside the cells, cholesterol is maintained within lipid droplets as cholesterol esters until ester hydrolase converts the cholesterol ester into free cholesterol. Further, mobilization of free cholesterol into the mitochondria is facilitated by a transport protein, steroidogenic acute regulatory protein (StAR). The internalization of free cholesterol from cytoplasm to the mitochondria is the rate-limiting step in the general steroidogenic pathway. Inside the mitochondria, free cholesterol is converted into pregnenolone by the cytochrome cholesterol side-chain cleavage (P450scc) enzyme. Pregnenolone is then transported to the smooth endoplasmic reticulum (ER) where it is converted to P4 by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) or to 17α-hydroxypregnenolone by the enzyme 17α-hydroxylase (Miller, 1988).

In theca cells, due to high 17α-hydroxylase activity pregnenolone is converted to 17α-hydroxypregnenolone. Sequentially, 17α-hydroxypregnenolone is converted to androstenedione by 17α-hydroxylase and 3β-HSD. In bovine theca cells, a very less amounts of androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD), and both androstenedione and testosterone are secreted. In GCs, androstenedione is first metabolized to estrone and subsequently to E2 and alternatively testosterone is also metabolized to E2 by aromatase (Wood and Strauss, 2002). Gonadotropin receptors, FSHR and luteinizing hormone/choriogonadotropin receptors (LHCGR) are predominant in granulosa and theca cells respectively. GCs of small follicles largely express receptors for FSH, though LHCGR are also moderately expressed in GCs of follicles >9 mm in diameter (Xu *et al.,* 1995). FSH stimulates the LHCGR levels, P450scc and aromatase activity, which lead to P4 secretion under LH stimulation. Simultaneously, the insulin/IGF-1 is also known to modulate steroid hormone production by increasing the expression of aromatase in responses to FSH stimulation (Armstrong *et al.,* 2002) . FSH *via* G-protein coupled receptors (GPCRs) stimulates adenylyl cyclase activity and increase production of cyclic adenosine monophosphate (cAMP) (Hunzicker-Dunn and Maizels, 2006). As cAMP signal is essential for FSH-dependent GCs differentiation (Grieshaber *et al.,* 2000).

IGF1 induces activation of protein kinase B (PKB) which is an important mediator of cell proliferation and survival (Mani *et al.,* 2010). Thus, steroidogenesis is dependent on the actions of both FSH and LH during the oestrous cycle and induction of ovulation.

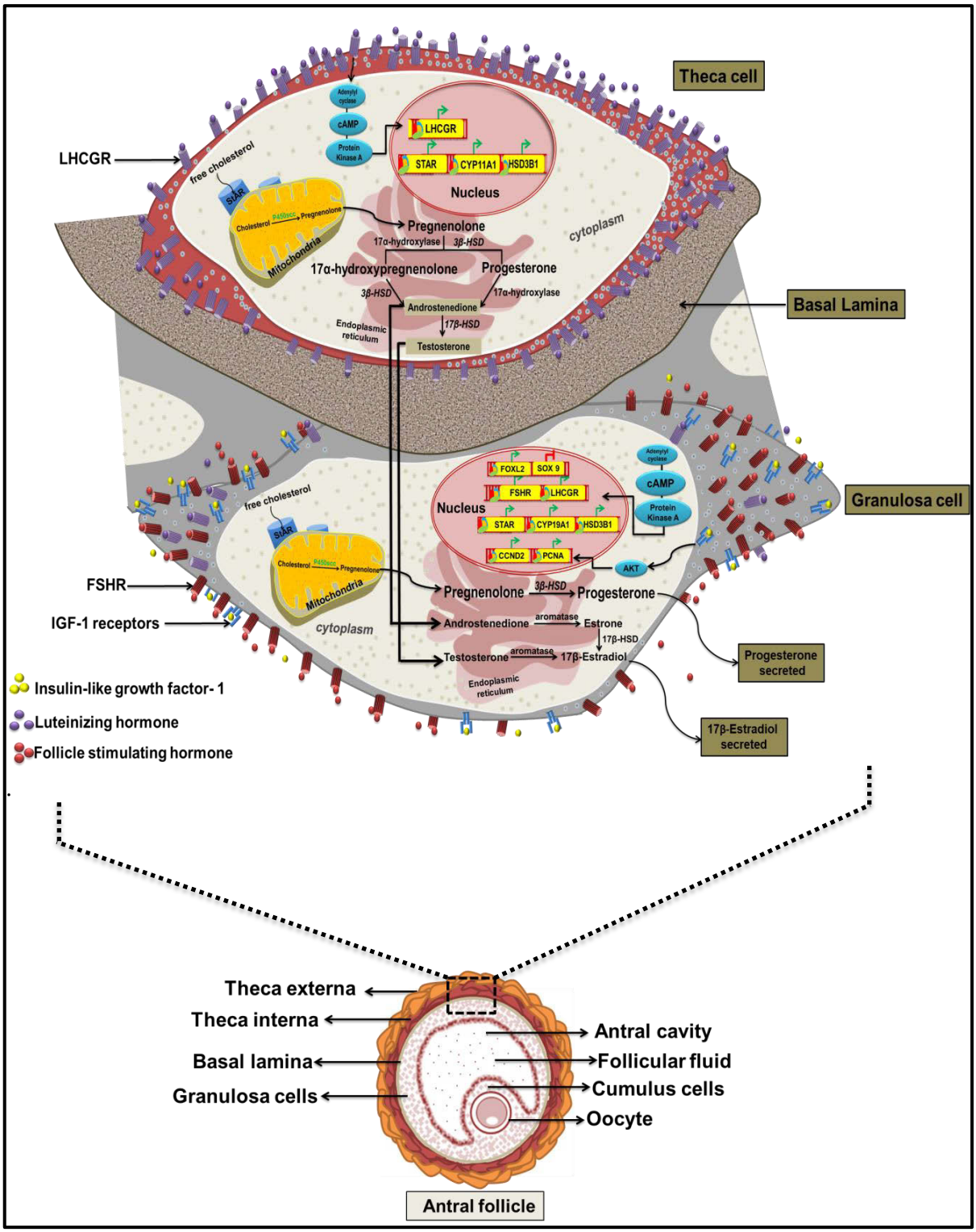

Figure 2. Steroidogenesis: Bovine theca and GCs interact during the follicular phase to synthesize E2 and P4, which are secreted into the FF and bloodstream. GCs actively display FSHR along with IGF-1 receptors and up-regulates genes involved in steroid biosynthesis.

1.2 Multiple stressors affecting ovarian function

The animal's response to stress conditions involves behavioral, physiological, cellular and subcellular reactions. A stress response can be either acute or chronic. The acute response is driven through the release of stress hormones like catecholamines and glucocorticoids, which can alter the metabolism and activate certain transcription factors at the cellular level. Whereas, the chronic response is driven by the endocrine system that involves modulation in the cell receptor population and change in homeostatic signals ultimately creating a new physiological state. Occurrence of such a new physiological state has been associated with the decline of immune competence, fertility and milk production in dairy cattle. Below, stressors such as heat stress, metabolic stress induced by energy imbalance and hypoxic stress that potentially affect and regulate the normal ovarian follicular physiology and functions in cattle are mainly described.

1.2.1 Thermal stress

Thermal stress during environmental heat stress conditions impairs follicular development in bovines. Cows with increased body temperature (hyperthermia) (40.5°C) show earlier emergence of a second dominant follicle, reduced numbers of medium sized follicles and low plasma E2 concentrations as compared to cows with normothermia (≤39.0°C) (Wolfenson *et al.,* 1995). Under gonadotropin stimulation, secretion of androstenedione and E2 is reduced in the follicles of heat stressed cows (Bridges *et al.,* 2005). Suppression of steroidogenesis in GCs with reduced aromatase activity, thus leading to low E2 production in the dominant follicle is also seen in dairy cows under seasonal and acute heat stress (Wolfenson *et al.,* 1997). Also, acute preovulatory heat stress leads to altered gene expression profiles in GCs of dominant follicles (Vanselow *et al.,* 2016). Oocyte developmental competence is quite dependent on follicular development, any perturbation inside the follicle will affect the oocyte. Oocytes retrieved from Holstein cows during summer season were reported to have low developmental competence leading to delayed embryo cleavage (Gendelman *et al.,* 2010). A higher proportion of oocytes when exposed to high temperature (41°C) *in vitro,* remained unfertilized, underwent apoptosis and had a disrupted nuclear maturation affecting the pattern of microfilament localization (Roth and Hansen, 2005). It was observed that embryos at later stages of development

(morula, blastocysts) were more heat resistant than embryos at initial stages (two-cell stage). Exposing *in vitro* produced putative zygotes to 41°C for 6h on days 0 and 2 after *in vitro* fertilization (IVF) was found to be more detrimental than the exposure to the same heat shock conditions on days 4 and 6 (Sakatani *et al.,* 2004). A functional CL is vital for embryonic development as it secrets adequate amounts of P4 to maintain pregnancy throughout the gestation period. However, cows exposed to chronic seasonal heat stress are reported to have impaired preovulatory follicles with a disrupted CL and as a consequence a substantial decrement in P4 production (Wolfenson *et al.,* 2002). Thus, it is evident that the ovarian components i.e. follicles, oocytes and CL are highly susceptible to elevated temperatures.

1.2.2 Metabolic stress: Lipid mobilization and elevated NEFA concentrations in blood and FF

A successful pregnancy, calving and higher lactation are most desirable metabolic outcomes in dairy cattle. However, the "*transition period*" i.e. 3 weeks before and 3 weeks after calving, are considered physiologically critical since during this period the animal is most susceptible to various metabolic and infectious challenges (Van, 2016). The early lactation period requires high energy intake for higher milk yield. However, feed intake during the transition period does not increase adequately or even declines thus resulting in a state of NEB. During NEB, high yielding cows selected for breeding undergo a significant loss of the body condition score and alterations in blood metabolite and hormone profiles, which in turn can influence fertility due to a decreased LH surge, low circulatory IGF-1, insulin, glucose and delay in resumption of ovarian cyclicity (Vicini *et al.,* 1991) (Butler, 2000) (Aungier *et al.,* 2014). During the initial weeks of parturition around 40% of dairy cows suffer from metritis, with ~21% showing symptoms of systemic illness like pyrexia. Also 15-20% of cattle suffer from clinical endometritis 3 weeks postpartum (Sheldon *et al.,* 2009). Embryo quality is reduced in lactating cows that undergo a reduction in body weight within 3 weeks postpartum thus leading to an increased rate of embryo degeneration (Carvalho *et al.,* 2014). Another risk factor due to NEB is excessive lipid mobilization which leads to elevated NEFA concentrations in the systemic circulation and ovarian FF in high yielding dairy cows.

Lipid mobilization: In order to compensate for the body energy demands excessive lipolysis of stored body fat occurs in postpartum cows leading to increased systemic concentrations of NEFAs like palmitic acid (PA), stearic acid (SA), oleic acid (OA) and of β-hydroxybutyric acid (BHBA) (Rukkwamsuk *et al.,* 2000). Triglycerides from adipose tissue are hydrolysed by a hormone-sensitive lipase to glycerol and free fatty acids (FFAs). Due to the lack of the enzyme glycerol kinase in bovine adipose tissues, glycerol cannot be further metabolized and is released as such into the circulation. FFAs are either re-esterified to form triglycerides or are released into the blood, bound to serum albumin. This release of FFAs from the adipose tissue is often termed as "*lipid mobilization*". Though a normal physiological process, chronic lipid mobilization may cause several metabolic disorders such as ketosis and fatty liver and reproductive disorders such as placental retention, metritis and endometritis (Roche, 2006). Elevated plasma levels of NEFAs, particularly of PA, SA, OA and linoleic acid (LA) are also well reflected in the FF due to their infiltration into the dominant follicle (Leroy *et al.,* 2005). This can affect both the COC morphology and embryo viability (Jungheim *et al.,* 2011) in bovine.

Elevated NEFAs in blood and FF: In the blood, fatty acids are present in form of triglycerides, cholesterol esters, phospholipids or as NEFAs, which are carried by serum protein albumin to various tissues. Numerous studies in humans, cattle, sheep and rodents have demonstrated the effects of changed fatty acid composition and proportion in serum and FF. Metabolic disorders such as obesity and type II diabetes in humans and NEB in cattle, are associated with upregulated lipolysis, leading to elevated NEFA concentrations in the serum (Karpe *et al.,* 2011). Differences in body mass index (BMI) are also associated with alterations in the fatty acid composition of the FF. In particular, differences in the abundance of the NEFA fraction were found in a BMI-related manner in FF of preovulatory follicles from normal weight ($18.5 \leq \text{BMI} < 25.0 \text{kg/m}^2$), overweight ($25.0 \leq \text{BMI} < 30 \text{kg/m}^2$) and obese women ($\text{BMI} \geq 30.0 \text{kg/m}^2$) undergoing assisted reproductive treatments. Gas chromatographic analysis of FF revealed significantly higher levels of NEFAs in obese women (315.53 ± 82.68 µmol/l) as compare to normal weight (221.67 ± 23 µmol/l) and overweight women (245.55 ± 35.98 µmol/l) (Valckx *et al.,* 2014). Such BMI-related variations of NEFA levels in FF could possibly affect GC viability, oocyte quality and subsequent embryo development possibly by acting directly on oocyte metabolism.

Lipid dysregulation in both seminal fluid and FF is also correlated to male and female fertility. Studies of seminal fluids of male infertile patients showed significantly elevated NEFA levels (11.4±0.097mg/dl) as compared to fertile males (7.8±0.078mg/dl). While in female patients undergoing IVF, NEFA levels were higher in serum (4.7±0.08 mg/dl) compare to control females (3.1±0.0.03 mg/dl) and alike, NEFA levels were higher in FF of female patients (6±0.1mg/dl) compared to control females (3.5±0.035 mg/dl) (Calonge *et al.,* 2018). Ketosis is another metabolic state which is also characterized by elevated NEFA levels. NEFAs were found increased in pregnant ewes with subclinical ketosis (1.02 mM) as compare to control (0.65 mM) ewes and ewes suffering from clinical ketosis (0.71 mM) (Marutsova and Marutsov, 2018).

NEFA concentrations in the blood and FF reflect the magnitude of NEB effects and lipid mobilization in dairy cows. Concentrations of NEFAs particularly of PA, SA and OA increase considerably in the circulation (Rukkwamsuk *et al.,* 2000). And these changes in serum NEFA concentrations are well reflected in FF of the dominant follicle (Leroy *et al.,* 2004a) (Leroy *et al.,* 2004b). The NEFA concentration in serum at 16 days postpartum was found significantly higher (0.4-1.2 mmol/l) as compare to the concentration at 44 days postpartum (0.1-0.3 mmol/l). A similar pattern was observed in FF with 0.2-0.6 mmol/l at 16 days postpartum compared to 0.1-0.3 mmol/l at 44 days postpartum. This clearly suggests that NEFA concentrations in both serum and FF are considerably higher during early as compared to subsequent postpartum periods (Leroy *et al.,* 2005). This phenomenon of sudden rise in NEFA levels was further observed in Holstein Friesian heifers exposed to a short period of fasting for 4 days. Interestingly, the concentration of OA was substantially higher in FF of fasted heifers, indicating that a relatively large proportion of OA is infiltrated into the FF during fasting (Aardema *et al.,* 2013). Liquid chromatography-mass spectrometry (LC-MS) detection of differential metabolites in cows with inactive ovaries showed higher plasma NEFA concentrations (0.32 ± 0.12 mmol/l) as compared to oestrous cows (0.21 ± 0.05 mmol/l) (Song *et al.,* 2019). This suggests that cows with inactive ovaries might experience higher lipid mobilization during the peak lactation period. Likewise, blood metabolic profiling revealed a higher NEFA concentration (0.71±0.04 mmol/l) in Holstein dairy cows with ovarian cysts as compare to cycling cows (0.43±0.07 mmol/l) (Jafari Dehkordi *et al.,* 2016).

Thus, these findings clearly suggest that the presence of elevated NEFA levels in blood might contribute to the development of abnormal ovarian phenotypes such as inactive ovaries with absent follicle cyclicity or ovarian cysts.

1.2.3 Stress induced by elevated NEFA levels

Elevated NEFA levels in FF can have detrimental effects on bovine GCs (Vanholder *et al.,* 2005). GCs as mentioned earlier are vital follicular cells responsible for the production of FF and plasma steroid hormones (Kulick *et al.,* 1999). Energy metabolism is vital for the oocyte to acquire developmental competence, which requires a lot of energy from various substrates such as carbohydrates, amino acids, and lipids (Collado-Fernandez *et al.,* 2013). This energy can be provided either from stored energy reserves inside the COC or external energy sources coming either from FF *in situ* or from culture media *in vitro*. FFAs are present in the FF and inside the COC in form of lipid droplets. Lipid droplets are considered as markers for healthy oocyte maturation in bovine. However, exposure of bovine COCs to elevated NEFA concentrations both *in vivo* and *in vitro* can lead to poor oocyte competence. But it is also observed that cumulus cells surrounding the oocyte are able to store fatty acids very well by forming lipid droplets, thus protecting the oocyte from direct exposure to elevated NEFA levels especially saturated fatty acids (SFAs). SFAs such as PA and SA reduces cell viability and induce apoptosis by down-regulating the apoptotic inhibitor B-cell lymphoma 2 (*BCL-2*) and up-regulating the apoptotic protein Bcl-2-associated X protein (*BAX*) in human GCs *in vitro* (Mu *et al.,* 2001).

IGF-1 signaling plays a vital role in FSH stimulation of PKB pathway and expression of steroidogenic genes in GCs. In bovine GCs, FSH acts synergistically with IGF-1 to increase the cell proliferation and aromatase expression (Mani *et al.,* 2010). Studies suggest that short dietary restraints in heifers lead to an anovulatory phenotype by reducing the systemic and follicular IGF-1 levels, which in turn lowers the messenger ribonucleic acid (mRNA) expression of cytochrome P450 family 19 subfamily A member 1 (*CYP19A1)* in GCs and *LHCGR* in theca cells, thus leading to decreased E2 synthesis (Walsh *et al.,* 2012). Also in mouse GCs, knocking down insulin-like growth factor 1 receptor (*IGF-1R*) leads to a reduction of *LHCGR* expression (Baumgarten *et al.,* 2017). Cystic ovarian follicles (COFs) may occur in lactating cows as a result of the disruption of insulin signaling. *IGF-1* mRNA expression was found high in follicular cells of cows with COFs in early and mid/late

lactation period, while higher mRNA expression of tumor necrosis factor (TNF) was found in cows with COFs in the mid/late lactation compared to control cows. Lower *CYP19A1* mRNA expression and higher plasma and follicular NEFA concentrations were only found in cows with COFs during the early lactation period. This indicates that COF pathogenesis may vary between early and mid-late lactation in dairy cows depending on the severity of NEB, which is predominantly higher during early lactation (Lima *et al.,* 2019). Elevated concentrations of NEFAs are also correlated with the polycystic ovarian syndrome (PCOS) in humans and rats. Obese PCOS patients undergoing IVF have higher PA and OA levels in both the plasma and FF as compared to control and non-obese PCOS patients. The frequency of deoxyribonucleic acid (DNA) fragmentation was also higher in embryos generated by IVF in PCOS patients irrespective of obesity. These findings suggest that the developmental competence is related to NEFA concentrations, which might contribute to the poor pregnancy outcomes in patients with PCOS. Similarly, high fat diet fed rats and PCOS rats had elevated FFA levels in the serum. The levels of prostaglandins and arachidonic acid metabolites were found significantly higher in ovarian tissues of PCOS rats, which could possibly restrain the ovarian cycle by inducing luteolysis (Niu *et al.,* 2014) (Huang *et al.,* 2018).

In bovine, the downstream effects of OA can be predicted from its inhibitory effects on both mRNA and protein expression of forkhead box protein L2 *(*FOXL2*)* in FSH induced cultured bovine GCs (Yenuganti and Vanselow, 2017). The deleterious effects of elevated NEFAs are also evident at the DNA level as observed in transcriptomic and epigenetic profiles of bovine blastocysts. NEFA exposure during oocyte maturation affects the expression of genes related to epigenetic programming such as histone H2B type 1-N (*HIST1H2BN*) involved in chromatin compaction and histone modification. Genome-wide analysis revealed that during oocyte maturation, elevated NEFA levels can influence the overall DNA methylation pattern in the resultant blastocysts affecting many differentially methylated regions (DMRs) related to cell death and survival (Desmet *et al.,* 2016). Also, down-regulation of DNA (cytosine-5)-methyltransferase 3A *(DNMT3A)* expression that is involved in *de novo* methylation of cytosine residues at CpG dinucleotides and differentiation in developing embryos has been observed in cumulus cells exposed to high NEFA concentrations (Van Hoeck *et al.,* 2013) (Uysal *et al.,* 2015). This suggest that

aberrant DNA methylation due to NEFAs can consequently lead to the development of less competent COC.

1.2.4 Hypoxic stress: a physiological "*eustress*"

"*Eustress*" means a beneficial physical, psychological or physiological stress. It is the positive response to a stress condition that is useful and contributes to the functionality of any mechanism. In bovine follicles, steroidogenesis, oocyte growth and maturation, ovulation and early events of CL development take place under reduced oxygen (O_2) tension in the GC layer and antrum. This suggests that follicle maturation has to take place under low O_2 conditions, which are often referred as "*hypoxia* " and may even operate as a positive stressor (Fischer *et al.,* 1992). Ovarian antral follicles consist of a vascularized peripheral theca cell layer separated by a basement membrane from the inner non vascular antral GCs layer. This structural barrier creates a hypoxic microenvironment for both antral GCs and COC**.** The O_2 released from the blood capillaries into the follicle first reaches the theca cells and then subsequently, lower amounts of O_2 diffuse *via* the basement membrane first into the mural GCs, then to antral GCs and finally to cumulus GCs surrounding the developing oocyte. This indicates that antral GCs along with the COC is exposed to hypoxia as the follicle grows. Studies suggest that the percentage of O_2 dissolved in FF is between 1-5% (Huey *et al.,* 1999), which is much lower than the atmospheric O_2 percentage (21%) used during normal *in vitro* culture conditions.

Hypoxia inducible factors (HIFs) have been proposed to be involved in follicle development perhaps due to the hypoxic microenvironment inside the follicle**.** HIF is a heterodimeric transcription factor consisting of one of an alpha (α) subunit and a beta (β) subunit. The HIF-α subunit has the O_2 sensitive domains and is induced by hypoxia, while the HIF-β subunit is constitutively expressed (Kumar and Choi, 2015). In HIF family, hypoxia inducible factor -1α (HIF1α) is most well recognized and a master regulator for the expression of genes involved in the response to hypoxia in mammalian cells (Semenza, 1999). At normal O_2 levels (20%), α subunit, which is O_2 sensitive, is rapidly ubiquitinated by prolyl hydroxylase. While at hypoxic conditions (0.1-1% O_2) HIF1α remains highly stable and conjugates with the HIF1β subunit, and is translocated to the nucleus, where it recruits transcriptional coactivators (Kallio *et al.,* 1998) for regulating the gene expression. This complex binds at enhancer domains of hypoxia responsive element (HRE) at either 3' or 5' regions of target genes

such as heme oxygenase-1, vascular endothelial growth factor (*VEGF*), glucose transporter 1 (*GLUT1*), and glucose transporter 4 (*GLUT 4)* (Pugh *et al.,* 1997). HIF-1α transcriptional activity is also known to be induced by FSH, inducing the up-regulation of *LHCGR*, inhibin-α and *VEGF* promoting follicular differentiation (Alam *et al.,* 2004). The existing hypoxic environment in preovulatory follicles plays an important role for luteinization of GCs as HIF1α expression promotes P4 synthesis in luteinized human (van den Driesche *et al.,* 2008) and bovine GCs (Yoshioka *et al.,* 2014). In preovulatory follicles, following the LH surge, cells of the theca and granulosa layers undergo luteinization thus displaying features that are quite different from those before the LH surge. And once the CL is fully developed, cells of the granulosa and theca cell layers become fully luteinized cells. A prominent feature of early luteinized GCs is the inhibition of cell proliferation, down-regulation of FSH signaling and steroidogenesis, up-regulation of HIF signaling and angiogenesis (Christenson *et al.,* 2013) (Wissing *et al.,* 2014). This suggests that existing hypoxia in preovulatory follicles play a vital role in luteinizing GCs to support the formation of a fully functional CL.

It is now well established that HIF1α up-regulation in preovulatory GCs is essential for ovulation. Inhibition of HIF activity by echinomycin effectively prevented ovulation in mice (Kim *et al.,* 2009). Genes such as endothelin-2 (Wang *et al.,* 2012), and *VEGF* (Duncan *et al.,* 2008) that are essentially involved in ovulation are up-regulated by HIF1 in preovulatory GCs. Hypoxia promotes vascularization by inducing *VEGF* expression in early bovine luteal cells thus facilitating CL formation (Nishimura and Okuda, 2009). It also stimulates the expression of *GLUT-1* thus facilitating an active uptake of glucose into the cells. Increased expression of *GLUT-1* has been found in early bovine luteal cells in response to hypoxia (Nishimura *et al.,* 2017). Dominant follicles increase in size during the post LH surge period, growing to a maximum diameter of 15-22 mm close to ovulation in bovines. With increasing diameters of the follicles, O_2 concentration decreases in the FF due to the increasing diffusion distance. Further, this fall in partial pressure of O_2 is accompanied with a drop in pH (potential of hydrogen) and an increase of partial pressure of carbon dioxide (CO_2) in the FF, indicating an active anaerobic respiration by follicular cells as indicated by increased glucose consumption and lactate accumulation in post-human chorionic gonadotropin (hGC) injected murine follicles (Sartori *et al.,* 2001) (Fischer *et al.,* 1992) (Harris *et al.,* 2007).

Thus, understanding the physiological "*eustress"* existing in form of hypoxia in preovulatory follicles is essential as it regulates the ability of the animal to establish pregnancy, which can later affect overall productivity.

1.3 Dietary lipids: a measure to improve ovarian function

Dairy cows are supplemented with dietary lipids to overcome energy deficiency during the NEB state and improve animal performance. The omega-3 and omega-6 fatty acids such as LA and alpha-linoleic acid (ALA) are essential fatty acids (EFAs) since mammals including bovine and humans cannot synthesize them *de novo*. Therefore, mammals need dietary supplementation of LA and ALA to produce higher order polyunsaturated fatty acids (PUFAs) such as arachidonic acid and docosatetraenoic acid (Tvrzicka *et al.,* 2011). However, limitation of feeding PUFAs diets to cattle is the extensive biohydrogenation of PUFAs in the rumen, which restrains them to reach the circulation. Though, the rumen also contains fungi and protozoans, but majority of biohydrogenation is performed by bacteria as compared to fungi and protozoa (Nam and Garnsworthy, 2007). Rumen bacteria such as Butyrivibrio *fibrisolven* hydrogenate the double bonds within the hydrocarbon chain of PUFAs to minimize their toxic effects by transforming them into saturated (mainly SA) or *trans* isomers of mono-unsaturated fatty acids (MUFAs) (Maia *et al.,* 2010). This prevents the actual proportion of supplemented PUFAs to be adequately absorbed by ruminants. In dairy cows, dietary lipids can be supplemented by abomasal infusion, in form of rumen-protected fat such as calcium salts of fatty acids and oilseeds and encapsulated lipids in formaldehyde treated protein coat. This helps supplemented lipids particularly PUFAs to bypass the ruminal biohydrogenation and increased the amount of PUFAs reaching the small intestine for absorption (Palmquist and Jenkins, 2017) (Barletta *et al.,* 2016).

In recent times, a better understanding of the role of dietary lipids in metabolic processes has provided a better chance to improve the reproductive efficiency in cattle. Fatty acids are involved in reproduction essentially as precursors for steroid hormones (*via* cholesterol) and prostaglandin (*via* arachidonic acid) biosynthesis. In bovine, increased size of the follicle has been positively correlated to improved oocyte competence (Lonergan *et al.,* 1994) Both dominant follicle size and CL volume increases in lactating cows fed with diets enriched in PUFAs compared to cows fed with a diet enriched in MUFAs (Bilby *et al.,* 2006). Diet enriched in high

omega-3/omega-6 ratio has been reported to increase the levels of LA and E2 in the follicle and improve the cleavage rate of IVF oocytes (Zachut *et al.,* 2010). High proportion of unsaturated fatty acids (UFAs) in cattle diet have been reported to increase the size and volume of preovulatory follicles with a higher androstenedione, E2 and E_2/P_4 ratio and increased aromatase expression in GCs. All these factors might help the cows to attain better conception rates (Zachut *et al.,* 2008) Diameter of the first dominant follicle, circulating IGF-1 and cholesterol levels at estrus have been found higher in cows fed with diets enriched in LA as compare to cows that did not receive the same diet. Whereas, cows fed with diets enriched in ALA increased the plasma E2 levels during the follicular phase. Also prior to luteolysis, 13, 14, dihydryo-15-keto-prostaglandin F2α (PGFM) released in higher amounts in response to oxytocin treatment in the cows fed with LA (Robinson *et al.,* 2002). Though, previous studies reported inhibitory effects of omega-3 PUFAs on prostaglandin synthesis (Arntzen *et al.,* 1998) (Coelho *et al.,* 1997). Nevertheless, this clearly suggests that the PUFA content of the diet can alter bovine ovarian and uterine function. Thus, metabolic conditions in dairy cows can be aided through dietary manipulation by inclusion of rumen protected lipid diets, helping lactating cows establish a more positive energy balance state and better oocyte development.

Overall, physiological alterations arising due to a deficient nutrient intake or energy balance can lead to altered hormonal responses in postpartum dairy cows, which is a major determinant of severity of NEB on subsequent fertility. Although these changes are normal adaptive process during the transition period in high yielding cows, failing to cope with these metabolic challenges such as high NEFA levels in serum and FF can profoundly modulate ovarian activity. These modulatory effects of increased NEFA levels particularly of PA, SA, OA, ALA and of *cis-9, trans-11* conjugated linoleic acid (CLA) and effects of hypoxia prevailing in preovulatory follicles on the functionality of cultured GC is extensively addressed and discussed in the presented dissertation. In addition, it is also addressed if dietary supplementation of EFAs to dairy cows can improve *in vitro* oocyte development competence.

2. Discussion

During the last decades, intensive breeding strategies have improved efficiency in dairy cows resulting in high milk yields. On the other hand, the transition period is still a major risk factor for subsequent reproductive performance in postpartum cows due to poor estrus expression and uterine tract infections (Wankhade *et al.,* 2017) (Opsomer *et al.,* 1996). Metabolically, both periods, pregnancy and lactation, are stressful to dairy cows. During the transition period, the ability of preovulatory follicles to synthesize sufficient E2 for the subsequent ovulation depends on the availability of metabolic hormones (insulin and IGF-1) in the serum and on the energy balance state (Butler, 2003). Metabolic hormones are crucial for postpartum ovarian follicular development as they interact with reproductive hormones (FSH and LH) to regulate normal ovarian activity (Sartori *et al.,* 2018). However, follicular growth disorders and transitory disturbances lead to inactive ovaries in postpartum cows (Peter *et al.,* 2009). One of the major metabolic changes in postpartum high yielding dairy cows are the increased concentrations of NEFAs in the serum and FF, which are positively correlated to NEB (Ospina *et al.,* 2010).

Initially, this dissertation highlights possible effects imposed by metabolic stress in the form of elevated NEFA concentrations on an important ovarian somatic compartment, the GC layer. Thus, systematic *in vitro* and *in vivo* approaches were used to investigate the effects of elevated NEFA concentrations on bovine GC function and ovulation. The follicular metabolism is not only dependent on endocrine factors but also on dietary lipids, which have emerged as new players in the regulation of the bovine ovarian function. This has also been addressed in the present dissertation where we assessed the effects of dietary EFAs on the *in vitro* developmental competence of oocytes obtained from German Holstein cows. Secondly, this dissertation also covers the effects of the rigorous physical conditions, to which growing follicles are exposed to. The inner non-vascularized somatic compartments of the ovarian antral follicles, consisting of metabolically active granulosa and cumulus cells are exposed to very low O_2 levels. This imposes an intriguing set of physiological challenges on the developing follicle and oocyte within it. However, prevailing evidence suggests that low O_2 levels, prevailing in the follicle, protect the oocyte from possible oxidative damage, while on other hand also provide

sufficient O_2 to the oocyte to meet its energy demands (Thompson *et al.,* 2015). Thus, low levels of O_2 elicit hypoxic responses in ovarian follicles especially with HIF-mediated gene expression, which is essential for the health of the follicle and residing GCs and COCs facing these conditions. Lastly, based on these facts we proposed that low O_2 levels contribute to stabilizing GC functionality. Briefly, data on effects of different fatty acids on the morphology, steroidogenesis, cell proliferation and cell viability of cultured primary GCs are presented in study I (PA, SA and OA) and study II (ALA and *cis-9, trans-11* CLA). In addition, study I also addresses *in vivo* effects in heifers following intrafollicular fatty acid injection, whereas in study II effects of dietary lipid supplementation on *in vitro* embryo production (IVP) are presented. The review article (study III) summarizes our own data and those of others on differential and partly opposing effects of SFAs and UFAs on somatic ovarian cells and oocytes of different species including humans with particular regard to metabolic diseases. Finally, effects of hypoxia on gene expression and hormone production in cultured GCs are described in studies IV and V.

2.1 Lipid accumulation in GCs: altered cell morphology and fatty acid transport

Intracellular lipid accumulation in form of lipid droplets exists from bacteria to humans (Zhang and Liu, 2017). Fatty acids cross the cell membrane *via* a transporter protein-mediated mechanism. Membrane-associated fatty acid binding proteins ('fatty acid transporters') not only facilitate but also regulate the cellular uptake of fatty acids. A number of fatty acid transporters have been identified, including cluster of differentiation 36 (CD36) , plasma membrane associated binding protein (FABP(pm)) and fatty acid transport proteins 1-6 (FATP1-6) (Schwenk *et al.,* 2010). *In vitro*, NEFAs are rapidly taken up by the cells from the medium and are either oxidized or incorporated into cellular lipid esters including phospholipids, cholesterol esters, and glycerides. However, when fatty acids are supplemented in excess, triglyceride synthesis and accumulation is greatly increased as evident from altered morphology and biochemical assays (Varinli *et al.,* 2015). CD36 is widely expressed in human, bovine and rat GCs (Osz et al., 2014, Wu et al., 2019). In studies I and II, we utilized *CD36* transcript abundance to characterize the fatty acid uptake by the cultured GCs after fatty acid supplementation. CD36 is an 88-kDa transmembrane protein which facilitates the fatty acid transport and is expressed in several mammalian cells such as adipocytes, enterocytes, muscle and endothelial cells (Kim and Dyck, 2016) (Glatz *et*

al., 2016). In addition to fatty acid uptake, CD36 also facilitates cholesterol uptake and transduces intracellular signaling that regulates the metabolic targeting of fatty acids (Pepino *et al.,* 2014). At the nuclear level, the expression of *CD36* is positively regulated by transcription factors such as nuclear peroxisome proliferator-activated receptors (PPARs). These are activated by fatty acids and regulate the transcription of genes involved in metabolism, inflammation, proliferation, and differentiation in various cell types (Neels and Grimaldi, 2014).

In the presented studies, *in vitro* supplementation of PA, SA and OA in study I, and of ALA and c*is-9, trans-11* CLA in study II, independently induced significant alterations in the morphology of cultured GCs. The altered morphology closely reminded of "*foam cells*", which are typical components of atherosclerotic plaques, where they actively participate in the intracellular cholesterol accumulation (Volobueva *et al.,* 2018). Additionally, these morphological changes were associated with a highly induced expression of *CD36* known to mediate FA uptake. In smooth muscle cells of apolipoprotein E-deficient ($ApoE^{-/-}$) mice exposed to OA, "*foam cell"* morphology was also described as CD36 dependent enhancing atherosclerotic lesions (Ma *et al.,* 2011). In mouse HC11 cells (mammary epithelial cell lines) OA induction of calcium (Ca^{2+}) ion and phosphoinositide 3-kinase (PI3K)/PKB signaling pathway was found CD36 dependent by suppression of these events by small interfering RNAs (siRNAs) (Meng *et al.,* 2018). Inhibition of *CD36* was also reported to attenuate adipocyte-induced cholesterol and lipid droplet accumulation and to reduce intracellular reactive oxygen species (ROS) production in human ovarian cancer cells (Ladanyi *et al.,* 2018). This suggests that loss of CD36 expression can protect cells from massive lipid droplet accumulation and can reduce lipotoxic effects. Our results presented in study I are also consistent with data of other studies in primary bovine mammary epithelial cells and in rat type II pneumocytes, where mRNA abundance of *CD36* was markedly increased by PA, SA, OA and LA (Yonezawa *et al.,*2004) (Guthmann *et al.,* 1999), thus suggesting that CD36 mediates the fatty acid uptake. An elevated level of PA uptake through CD36 up-regulation is well associated with the induction of epithelial-mesenchyme transition (EMT) in human hepatoma cells, HepG2 or Hep3B. PA causes lipotoxicity and increases the mRNA expression of EMT transcription factors causing progression of EMT (Nath *et al.,* 2015). PA was also found to induce inflammatory genes in macrophages *via CD36* expression as

evidenced by inhibition of CD36 with sulfosuccinimidyl oleate (SSO). SSO attenuated the PA induced lipopolysaccharide stimulated gene expression (Lu *et al.,* 2017). SA accumulation in human GCs is known to induce apoptosis. Intracellular SA accumulation in mouse peritoneal macrophages (MPMs) induces c-Jun N-terminal kinases (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated inflammatory pathways, thus resulting in ER stress mediated apoptosis (Anderson *et al.,* 2012).

Study II also showed that both, ALA and *cis-9, trans-11* CLA treatment, induced intracellular lipid droplets in GCs particularly at higher concentrations. This was again associated with an increased transcript abundance of *CD36*. The morphology of GCs after ALA and *cis-9, trans-11* CLA treatment was quite similar to that reported earlier (study I) in GCs under PA, SA and OA treatment. The observed results in study II are consistent with reports in other mammalian cell lines. c*is-9, trans-11* CLA induced lipid accumulation in human macrophages and in mice 15P-1 cell lines (testicular cells) along with an elevated expression of the *CD36* gene. *cis*-9,t*rans*-11 CLA and LA up-regulated *CD36* gene expression and induced lipid deposition in human macrophages/foam cells (Stachowska *et al.,* 2010). The consumption of an ALA enriched diet increased the skeletal muscle content of ALA and EPA and also increased the rate of PA transport into the sarcolemma as evident from the increased sarcolemmal CD36 protein expression (Chorner *et al.,* 2016). Feeding an ALA enriched diet to mice protected them from alcoholic hepatic steatosis as dietary flaxseed oil supplementation effectively increased *CD36* expression while decreasing the ethanol-induced hepatic lipid accumulation (Wang *et al.,* 2016). This suggests that both ALA and *cis-9, trans-11* CLA are well-accumulated in mammalian cells either *in vitro* or *in vivo* as dietary supplements. Levels of CD36 protein have been found higher in GCs from obese than normal weight females. E2 secretion and GC proliferation was inhibited along with increased apoptosis in GCs of obese women. These effects could be attributed to increased cellular lipid peroxide levels arising due to high accumulation of triglyceride in GCs (Wu *et al.,* 2019). This suggests that CD36 overexpression in GCs of obese women is one of the mechanisms by which obesity can impair GC function in females.

2.2 Elevated FFAs modulate steroidogenesis in GCs and affect ovulation

As already explained the biosynthesis of steroid hormones "*steroidogenesis*" is an integral function of bovine GCs. Together, in study I and II we observed that SFAs (PA and SA) and UFAs (OA, ALA and *cis-9, trans-11* CLA) clearly modulate GC steroidogenesis differentially (see below). This is most probably due to different degrees of saturation or stereo-metric positions of double bonds present in the respective fatty acids. Differential effects of SFAs and UFAs have also been reported previously in *in vitro* and *in vivo* studies (Uddin et al., 2017, Tamer and Nergiz-Unal, 2017).

2.2.1 SFAs stimulate steroidogenesis and cell proliferation

In study I, stimulatory effects of SFA supplementation on steroidogenesis of cultured GCs were observed. E2 concentrations were higher in conditioned media after PA and SA supplementation. These higher levels of E2 in PA and SA treated GCs were further confirmed by gene expression analysis. Interestingly, the key genes of steroidogenesis *CYP19A1* and hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase 1 *(HSD3B1)* were significantly up-regulated along with higher expression of gonadotropin receptors (*FSHR* and *LHCGR*). FSH is known to induce the expression of *LHCGR via* protein kinase A (PKA) and PI3K pathways as also shown in rat GCs (Law *et al.,* 2013). *LHCGR* is also highly expressed in GCs during the preovulatory stage increasing the responsiveness to the preovulatory LH surge required for oocyte maturation, ovulation and CL formation. Thus, the observed up-regulation of both *FSHR* and *LHCGR* in PA and SA treated GCs might therefore enhance FSH signaling thus leading to the observed stimulatory effects on steroidogenesis. However, the expression of *STAR* was significantly down-regulated by both PA and SA, thus rather implying a reduction of E2 production. The observed opposite effect might be due to the presence of androstenedione in our GC cell culture medium. Androstenedione is a preferred substrate for aromatase to produce E2. Our results on stimulatory effects of PA and SA on E2 production are in line with previous findings by others where exposure of SFAs to bovine androgen producing cells significantly increased the androgen production (Bellanger *et al.,* 2012). Also in mouse GCs, *CYP19A1* expression was found to be elevated by SA supplementation (Valckx *et al.,* 2014).

The flow cytometry data shown in study I revealed that despite morphological alterations induced by PA and SA due to lipid accumulation, these fatty acids had no noticeable apoptotic effects. In contrast, PA and SA have been reported earlier to induce apoptosis in human GCs (Mu *et al.,* 2001) and to cause PA induced lipoapoptosis in rat exocrine pancreatic AR42J cells (Landau *et al.,* 2006) through up-regulation of pro-apoptotic *BAX* and down-regulation of anti-apoptotic *BCL2* expression. To find a possible explanation for these contradictory results it is necessary to consider the different players affecting apoptotic events in more detail.

In multicellular organisms, both cell proliferation and cell death are tightly regulated to maintain tissue homeostasis. Cyclin D2 (*CCND2*) gene plays an important role in the induction of gap-1-synthesis (G1-S) phase transition and is required for GCs proliferation during ovarian folliculogenesis as suggested by knockout studies in mice (Burns *et al.,* 2001) (Santamaria and Ortega, 2006). In study I, both PA and SA had stimulatory effects on E2 production along with an up-regulation of *CCND2*. In cattle, it is well known that follicles, which secrete higher E2 levels continue to grow till they reach the ovulatory stage (Evans and Fortune, 1997). Since higher E2 levels stimulate both, GC proliferation and differentiation (Couse et al., 2005, Drummond et al., 2002), elevated E2 levels are positively correlated with GCs proliferation in bovine. Higher E2 levels protect GCs from Fas ligand-induced apoptosis and increase the percentage of cells entering the G1-S phase transition of the cell cycle, which is associated with an increased *CCND2* protein expression (Quirk *et al.,* 2006). This suggests that E2 levels might influence apoptosis in GC by stimulating the G1-S phase transition as it is evident from higher expression of *CCND2* and thus protecting GCs against SFA induced apoptosis. Additionally, the chemical properties of different fatty acids (e.g. saturated or unsaturated) also affect the membrane integrity of the cells and their ability to penetrate through the cell membranes, which might affect E2 biosynthesis. In any case, increased E2 production that is induced by SFAs on one hand might counteract the pro-apoptotic effects of these fatty acids on the other hand. This suggests that differential E2 production by GCs must be taken into account when comparing different cell culture models.

2.2.2 UFAs alter steroidogenesis, expression of identity markers, cell proliferation and apoptosis in GCs

2.2.2.1 Steroidogenesis and identity markers

We determined the effect of the UFAs, OA (study I), and ALA and *cis-9, trans-11* CLA (study II) on steroid hormone production and on transcriptional activity of key genes involved in gonadotropin hormone signaling (*FSHR* and *LHCGR*), steroidogenesis (*CYP19A1, STAR* and *HSD3B1*) and GCs identity markers (*FOXL2* and SRY (sex determining region Y) - box 9 (*SOX9*)) in cultured GCs. Since both, folliculogenesis and steroidogenesis are closely dependent on the coordinated actions of FSH and LH with their receptors on GCs and theca cells of ovarian follicles. E2 production is essential for a progressive folliculogenesis as evident from studies in aromatase gene knockout (ArKO) mice. ArKO females develop ovaries with follicles that are arrested before ovulation without CL formation and almost negligible P4 levels (Fisher *et al.,* 1998). In our experiments described in studies I and II the transcript levels of *STAR*, *CYP19A1* and *HSD3B1* along with E2 production were significantly reduced under OA, ALA and *cis-9, trans-11* CLA supplementation in cultured bovine GCs. The reduction in E2 concentration can be most likely attributed to a reduced transcription of the *CYP19A1* gene that is essential for converting androgens into estrogens.

However, previous studies have observed stimulatory effects of OA (500µM) on E2 production (Vanholder *et al.,* 2005). In this study the authors harvested GCs from large follicles (>8mm) and cultured them just for 48 hours whereas in our GC culture model the cells were derived from small follicles and were cultured for 8 days. Another reasonable explanation could be that GCs from large follicles secrete (295 ± 6 pg/day/10^4 cells) more E2 than GCs from medium follicles (44.7 ± 6 pg/day/10^4 cells), which in turn secrete twice as much E2 as GCs from small follicle (24.04 ± 6 pg/day/10^4 cells) (Gutierrez *et al.,* 1997). However, also in buffalo, cultured GCs also reduce *CYP19A1* expression and E2 production in the presence of CLA (Sharma and Singh, 2012). Arachidonic acid, another long chain fatty acid stimulates bovine GC proliferation by activating both extracellular signal-regulated kinases 1/2 (ERK1/2) and PKB at 50 µM. However, higher doses decreases E2 secretion and down-regulated the mRNA abundance of *CYP19A1, FSHR*, *HSD3B1* and *STAR* in cultured bovine GCs (Zhang *et al.,* 2019). This suggests that fatty acids might modulate

steroidogenesis by specific intracellular signaling pathways that regulate target gene expression. Our results suggest that the observed down-regulation of *FSHR* and *LHCGR* by UFAs at higher concentrations might affect FSH signaling and steroidogenesis in bovine GCs. P450scc enzyme converts cholesterol into pregnenolone and 3β-HSD enzyme converts pregnenolone into P4. 3β-HSD is encoded by *HSD3B1* gene whose expression however, remained unaltered in GCs treated with ALA, while the P4 production was reduced. Similar effects on P4 production have been observed in primary goat GCs treated with ALA at 100 μM (Coyral-Castel *et al.,* 2010). Surprisingly, *cis-9 trans-11* CLA significantly up-regulated *HSD3B1* gene expression, while reducing the P4 concentration in conditioned media. This outcome might be due to negative feedback effects of P4 on *HSD3B1* transcription. Nonetheless, together our results suggest that high levels of UFAs preferentially of OA, ALA and *cis-9, trans-11* CLA in FF can adversely affect GCs by reducing both E2 and P4 production, which in turn could result in compromised ovarian cyclicity and impaired fertility in lactating cows.

FOXL2 is another major transcriptional regulator of GC differentiation and folliculogenesis. It is required to maintain GC identity in females (Georges *et al.,* 2014). Studies in *FOXL2* knockout mice models have shown its contribution to GC differentiation (Schmidt *et al.,* 2004). *FOXL2*, suppress the expression of *SOX9*, the transcriptional regulator of sertoli cells in testes (Uhlenhaut *et al.,* 2009). *FOXL2*$^{-/-}$ null female mice cannot form ovarian follicles (Uda *et al.,* 2004) and up-regulated the genes that were required for male sex determination such as *SOX 9*, splicing factor 1 (*SF1)* and GATA Binding Protein 4 (*Gata4)* (Ottolenghi *et al.,* 2005). It was already reported that OA at higher concentration (400 μM) suppressed *FOXL2* and induced *SOX 9* expression both at the transcriptional and protein level in cultured bovine GCs (Yenuganti and Vanselow, 2017). At the transcriptional level, we observed a similar regulation of *FOXL2* and *SOX 9* genes in study II, where GCs treated with ALA and *cis-9, trans-11* CLA decreased the mRNA abundance of *FOXL2* transcripts significantly. Apart from its role as a key GC identity marker, *FOXL2* is also a well-known regulator of ovarian steroid metabolism. In mouse ovaries, FOXL2 interacts with ESR1 and regulates the function of key gene involved in estrogen production (Uhlenhaut *et al.,* 2009). FOXL2 also induces the expression of follistatin, which is involved in the progression of follicles from antral to dominant stages (Kashimada *et*

al., 2011). Thus, the results presented in study II clearly indicate that *FOXL2* transcriptional activity can be modulated by PUFAs such as ALA and *cis-9, trans-11* CLA. Any disruption of normal *FOXL2* function in GCs due to high NEFA levels in the circulation or FF might prove instrumental in altering the key genes of steroidogenesis.

2.2.2.2 Cell proliferation and apoptosis

Cell cycle regulators and apoptotic factors influence the fate of cells in multi-cellular organisms in order to regulate homeostasis as evident by studies on *c myc* proto-oncogene (Pucci *et al.,* 2000). The flow cytometry data of study II showed that OA at higher concentrations despite suppressing the *CCND2* gene expression did not induce any signs of apoptosis in cultured GCs. This is in line with earlier studies reporting that OA does not induce apoptotic effects on cell lines such as Chinese hamster ovary (CHO) cells, but instead protects the cells from PA induced apoptosis (Listenberger *et al.,* 2003). Morphological observations of OA treated GCs indicate that OA causes lipid accumulation more effectively as compared to SFAs such as PA and SA. This suggests that OA is incorporated as triglyceride more effectively and is less apoptotic as compared to SFAs, which are poor substrate for triglyceride synthesis. Both ALA and *cis-9, trans-11* CLA treatment of cultured GCs down-regulated the *CCND2* mRNA expression. However, considering cell viability, only *cis-9, trans*-11 CLA elicited significant apoptotic effects in cultured GCs. Previous studies showed that CLA induces apoptosis in human (Wang *et al.,* 2008), mouse (Ou *et al.,* 2007) and rat cells (Ip *et al.,* 2000).

In bovine mammary cell-line (Mac-Tcells) at high doses, CLA isomers have detrimental effects on cell growth and survival *in vitro* with increased DNA fragmentation as compared to bovine serum albumin (BSA) control (Keating *et al.,* 2008). In GCs, the *CCND2* mRNA expression rapidly increases in response to FSH stimulation (Han *et al.,* 2013), whereas FSH β-null mutant mice show decreased *CCND2* mRNA (Burns *et al.,* 2001). This is also well in line with our observation of a reduced E2 production and reduced *FSHR* mRNA expression followed by a steep down-regulation of *CCND2* mRNA expression in *cis-9, trans-11* CLA treated GCs. However, ALA despite reducing the E2 levels and *CCND2* mRNA abundance did not induce any apoptotic effects in GCs. These earlier observations are well in line with our observation in OA treated GCs, where *FSHR* mRNA expression was significantly

decreased, thus reducing the FSH responsiveness. Considering this, the down-regulation of the *CCND2* transcript abundance as observed in ALA and *cis-9, trans-11* CLA treated GCs might be a consequence of reduced FSH signaling due to UFA treatment. These observations are true indicators for the importance of FSH signaling for GC proliferation. Another important regulator of cell proliferation is proliferating cell nuclear antigen *(PCNA)* gene which remained unaltered in its mRNA expression in GCs treated with ALA and *cis-9, trans-11* CLA. However, data from previous immunocytochemistry provide evidence for inhibitory effects of *cis-9,trans-11* CLA on PCNA expression , cell growth and proliferation in mammary cancer cells (MCF-7 cells) at different time periods (Liu *et al.,* 2002b) and in gastric adenocarcinoma (SGC-7901) cell lines (Liu *et al.,* 2002a). Accordingly, we might speculate that PCNA at the protein level can be altered due to *cis-9, trans-11* CLA treatment. Though, in our study *PCNA* expression was measured only at transcription level, but not at the translation/protein level.

2.2.3 Combined effects of NEFA (PA+SA+OA) concentrations on GC function

In study I, cumulative effects of higher physiological concentrations of PA (200µM), SA (200µM) and OA (400µM) were tested on *in vitro* cultured GCs. Combined supplementation of (PA+SA+OA) induced significant morphological alterations in the cells. The altered morphology closely reminded of the "*foam cells*" morphology as observed during treatment with individual fatty acids already discussed above. Additionally, as expected the mRNA expression of *CD36* was significantly higher in GCs indicating an active uptake of fatty acids. Real time-quantitative polymerase chain reaction (RT-qPCR) analysis of key transcripts of steroidogenesis (*CYP19A1*, *HSD3B1),* of gonadotropin receptors *(FSHR* and *LHCGR)* and of cell proliferation *(CCND2* and *PCNA)*, revealed no significant changes. The E2 production was though significantly reduced suggesting that the effect of OA might dominate in the supplemented fatty acid mixture. Contrary to these results, previous studies on cumulative effects of PA, SA and OA reported lower cell numbers and increased E2 production in cultured bovine GCs (Vanholder *et al.,* 2005). In theca cells however, the same authors reported a significant reduction of proliferation along with a higher percentage of cells undergoing apoptosis and a decreased P4 production as compared to controls (Vanholder *et al.,* 2006). Apoptotic effects of SFAs are well reported in human and bovine GCs as already discussed, but there were no signs of apoptosis in

GCs treated with combinations of PA+SA+OA, though the percentage of viable cells (29.7±1%) was significantly lower than in BSA treated controls (55.7±9.5%). The reduced percentage of viable cells observed in combination treatments was probably due to cytotoxic effects imposed by NEFAs. Earlier observations have shown that OA effectively rescued SFA induced apoptosis due to the activity of the enzyme stearoyl-CoA desaturase 1 (SCD1) that catalyzes the conversion of SFAs into MUFAs, thus directing the saturated NEFAs into triglycerides, escaping the apoptotic pathway (Matsui *et al.,* 2012). So, it can be speculated that exposure of GCs to PA+SA+OA either resulted in a higher percentage of dead cells due to non-apoptotic cell death pathways (Tait *et al.,* 2014) or as reported in other studies, to insignificant changes of cell numbers as discussed earlier.

Studying combined effect of fatty acids *in vitro* provided us to better understand the *in vivo* situation where GCs are exposed to mixed pools of fatty acids. *STAR* expression was down-regulated by all three fatty acids individually or in combined treatment experiments. This suggests that elevated *in vivo* concentrations of mixed fatty acids during NEB might cause cumulative effects on *STAR* expression in steroidogenic cells similar to our *in vitro* observations. In addition, the inhibitory effects of OA might supersede the stimulatory effects of PA and SA, thus leading to a significant reduction of *in vitro* E2 production after combination treatment. According to previous studies, OA is also found at higher concentrations in FF of cows facing short term energy crisis (Aardema et al., 2015). Increased serum NEFA concentrations can very likely initiate pro-inflammatory responses in cows during the transition period. mRNA abundance of proinflammatory cytokines like interlukin-6 (IL-6) and interlukin-8 (IL-8) and adhesion molecules such as intercellular adhesion molecule 1(ICAM1) and vascular cell adhesion protein 1 (VCAM1) was increased remarkably in bovine aortic endothelial cells (BAECs) after treatment with NEFA mixtures (Contreras *et al.,* 2012). Similarly, exposing human monocytic leukemia (THP-1) cells to NEFA mixture *in vitro* enhances their adhesion to vascular endothelium through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, increased ROS generation and increased mRNA expression of cluster of differentiation 11 b (CD11b) (Zhang *et al.,* 2006). Intense lipid mobilization as observed during the transition period in dairy cows might lead to alterations in inflammatory responses associated with increased disease susceptibility. Thus, these observed effects of treatments with combined NEFAs (PA+SA+OA) on

steroidogenesis and cell viability of cultured GCs could be possibly involved in early postpartum ovarian dysfunction.

2.2.4 Elevated OA levels impede ovulation

To validate the *in vitro* data of detrimental effect of OA on cultured GCs, we tested the effects in an animal model by directly injecting OA or BSA vehicle controls into the growing dominant follicles. This intrafollicular injection approach avoided difficulties to control systemic effects that can be assumed during fasting experiments, but instead allowed the exposure of antral GCs directly to the injected OA concentration (400 μM). In some heifers, the post-injection ovulation rate was monitored, whereas in others, injected follicles were aspirated 48 hours later in order to collect FF and GCs therein for hormone estimation and gene expression analysis. From 14 injected growing dominant follicles (one per animal) that were kept for monitoring the ovulatory competence, nine were injected with BSA vehicle control, out of which seven follicles advanced to the ovulatory stage (77%), while only one out of five follicles injected with OA ovulated (20%) within 96 hours post-injection. Although not significant yet the ovulatory competence tended to be reduced following the OA as compared to the BSA injections. In earlier studies by others, the intrafollicular injection approach was used to study the *in vivo* effects of IGF-1 in follicle deviation in heifers and mares (Ginther *et al.,* 2004). Our *in vivo* data clearly provided an affirmation of the results obtained *in vitro*. As discussed above, *in vitro* data indicated that OA has a dominant effect in combination treatment as evident by significantly lower amounts of E2 produced in combination treatments (PA+SA+OA). Therefore, we specifically selected OA for intrafollicular injection.

To validate this elaborate experimental approach, gas chromatography analysis of FF samples obtained 48 hours post-intrafollicular injections were used. The data revealed that the percentage of OA (46.5±5.8%) in injected follicles was significantly higher as compared to BSA (13.6±2.5%) injected follicles, whereas the proportions of a majority other fatty acids were not markedly altered (see Annexure 2). These *in vivo* results were consistent with those reported *in vitro*, as E2 concentrations were significantly reduced in FF recovered from follicles that were injected with OA. Since, dominant follicles normally secret adequate amounts of E2 to induce the preovulatory LH surge, thus the observed reduction in E2 concentrations in FF of OA injected follicles might have caused lower ovulation rates. Furthermore, gene

expression analysis in GCs recovered from OA injected follicles revealed higher *CD36* mRNA abundance along with significantly lower *CYP19A1* and *STAR* mRNA abundance as compared to GCs recovered from BSA injected follicles. Thus, *in vivo* results evidently indicated that following intrafollicular injection, OA was actively transported into GCs by *CD36* being up-regulated and both, *CYP19A1* and *STAR* down-regulated 48 hours post OA injection. Since, *CYP19A1* is the key enzyme of E2 synthesis the resulting lower *in vivo* concentration of E2 in the FF is evidently in line with suppressed *CYP19A1* expression. Thus, considering these data we could anticipate that OA, which is mobilized during the period of NEB postpartum, could considerably affect GC steroidogenesis and ovulation in dairy cattle.

From our data, it became very clear that SFAs (PA and SA) and UFAs (OA, ALA and *cis-9, trans-11* CLA) elicit differential effects on GCs with SFAs being stimulatory and UFAs being inhibitory on both hormone production and the abundance of key transcripts. GCs remained largely under the influence of OA in the *in vitro* combined supplementation with PA+SA+OA. This could give us an idea about effects on GCs *in vivo*, when the OA concentration is prominently higher in mixed pools of fatty acids in the follicular antrum due to an extensive NEFA mobilization from adipose tissue. Additionally, the *in vivo* approach of intrafollicular OA injection confirmed our assertion that OA can impede steroidogenesis in GCs and the ovulatory competency of dominant follicles. Collectively, these inhibitory effects of NEFAs, especially of UFAs might be among the causes through which NEB influences the reproductive performance in high-yielding dairy cows during the early postpartum period. Positively, we speculate that these findings may serve instrumental in understanding fertility issues in obese women with increased serum NEFA concentrations suffering from impaired fertility.

2.3 Dietary lipids and IVP in bovine

Over the past decades, a substantial increment in performance has been observed in dairy cattle due to improved herd management, nutritional diets and genetics (Miglior *et al.,* 2017). Modern dairy diets are often supplemented with rumen-protected fat to increase the energy intake in the early postpartum period and to increase fertility in dairy cows. Fat supplementation not just increases the energy balance but also increases the overall dietary energy content, which stimulates milk production (Lanier and Corl, 2015). Dietary supplementation with rumen-protected omega-3 fat sources

to cows is well reflected in the fatty acid profile of plasma and FF (Childs *et al.,* 2008) , and has been related to improved fertility (Santos *et al.,* 2008).

In study II, which was part of a large cooperative dietary study (FITCOW, PTBLE, Geschäftszeichen: 313-06.01 -28-1 -79.003-15) on the effects of dietary EFA supplementation on German Holstein cows during the transition period. The effects of four different abomasal supplemented dietary lipids on IVP were analyzed. For supplementation, cows in their 18th week of gestation and 2nd lactation received dietary rations including (i) coconut oil as control (CTRL), diet (ii) linseed and safflower oil as a source of EFA, (iii) Lutalin® as a source of CLA and (iv) a mixture of linseed and safflower oil plus Lutalin® (EFA+CLA) (see Annexure 3 and 4). Nine weeks postpartum the cows were slaughtered and the FF from large follicles 10-24 mm and oocytes were collected by slicing the ovaries. Gas chromatography analysis of FF showed a higher percentage of ALA in the FF of EFA and EFA + CLA diet fed cows, while the proportion of *cis-9, trans-11* CLA was higher in the FF of CLA and EFA+CLA diet fed cows as compared with cows fed with the CTRL diet.

Previous studies suggest that FA composition of plasma and ovarian compartments undergo modifications in response to fat supplementation. The encapsulated fat containing flaxseed oil increased the proportions of ALA in both plasma, milk fat, FF and GCs. Also, the number of follicles and the oocyte cleavage rate was higher in cows that were fed with a flaxseed oil diet as compared to control diet cows. Thus indicating that dietary omega-3 fatty acids can enrich the FF and can further increase the cleavage rate of oocytes (Zachut *et al.,* 2010). Similarly, other studies showed that the proportion of ALA was greater in FF, GCs and COCs of flaxseed oil fed cows as compared to cows fed with diets containing fish oil and saturated fat. Follicle numbers and oocyte cleavage rates were higher in flaxseed oil fed cows and fish oil fed cows than in saturated fat fed cows. Also, the percentage of oocytes that developed to blastocysts tended to be higher in cows fed with flaxseed oil and fish oil as compared to saturated fat (Moallem *et al.,* 2013). This suggests that supplementing dietary EFAs to dairy cattle may present a satisfactory approach to improve oocyte quality as it improved folliculogenesis and IVF performance.

In our study, we observed increased proportions of ALA and *cis-9, trans-11* CLA in the FF of cows in the EFA, CLA, and EFA + CLA dietary groups. However, this did not lead to any significant improvement in ovarian performance. Our results revealed that neither EFA, CLA nor EFA+CLA dietary supplements could improve

the cleavage, blastocyst rates or total cell number (TCN) of *in vitro* generated embryos as compared to the CTRL diet group. These results are in line with previous studies that have also reported negligible effects of PUFAs diet in Holstein cows. Feeding PUFA enriched diets, in spite of improving the size of preovulatory follicle and CL volume, failed to improve oocyte nuclear maturation, oocyte quality and embryo development *in vitro* (Bilby *et al.,* 2006). Negligible effects of diets enriched in soybean (high in LA), linseed (high in ALA) on developmental competence of oocytes have been observed in lactating dairy cows. Though fatty acid profiles in plasma and milk well reflected the profiles of dietary FA sources, dietary plasma concentrations of metabolic hormones such as, insulin, IGF-1 and leptin remained unaltered. Also cows fed with soybean or linseed did not show any improvement either in the blastocyst rate or TCNs of *in vitro* produced embryos compared to rumen inert fat (RIF) diet fed cows (Fouladi-Nashta *et al.,* 2009). These observations are partly in line with our results as both blastocyst rates and TCN counts in Hoechst stained blastocyst generated from each dietary groups remained unaltered. Previous findings have utilized caspase activity and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays to determine apoptosis in embryos generated from cows fed with diets containing MUFA or PUFA. PUFAs such as arachidonic acid, ALA and LA are known to suppress apoptosis in W256 carcinoma cells (Tang *et al.,* 1997). However, in study II we did not determine apoptosis in *in vitro* produced embryos as TCN in blastocyst is also one of the criteria for assessing blastocyst quality (Ushijima *et al.,* 2008).

Apart from dietary supplementation, *in vitro* studies have provided new insights into the oocyte developmental competence by directly exposing the oocytes to media containing different long chain fatty acids. In pig, it was reported that ALA (25μM) supplementation during *in vitro* maturation (IVM) increased the oocyte cleavage rate. However, though ALA enhanced the nuclear maturation of oocytes at higher dose (50μM), it could not improve the cleavage or blastocyst rates, or TCN counts. This suggests that *in vitro* higher concentration of ALA can adversely affect the embryo development (Lee *et al.,* 2017). Higher doses (50 to 200 μM) of *cis-9, trans-11* or *trans-10, cis-12* CLA during *in vitro* culture (IVC) decreased the development competence of activated parthenogenetic embryos in a dose dependent manner. However, embryos generated under *cis-9, trans-11* CLA (100μM) before vitrification were of better quality. Adding 100 μM of *cis-9,trans-11* CLA during the

last 36 hours to the IVC medium increased the survival rate and blastomere count in vitrified embryos as compare to control vitrified embryos (Absalón-Medina *et al.,* 2014). CLA was also reported to protect oocytes from damage occurring throughout the vitrification processes. Supplementation of oocyte culture medium with CLA improved the rate of viable COCs and cleavage rates. A slower influx of the 10 % ethylene glycol and 10 % dimethyl sulfoxide cryoprotectant solution was observed in oocytes matured in the presence of CLA. This suggests that CLA could be incorporated in the oocyte membrane and thus affect membrane fluidity (Matos *et al.,* 2015). In another study it was reported that *in vitro* ALA supplementation can also protect oocyte developmental competence against the lipotoxicity caused by PA, SA and OA by protecting cumulus cell viability. ALA in the presence of PA, SA and OA was able to control embryo fragmentation and improve cleavage, blastocyst rates and blastocyst quality. Also, ALA in the presence of PA, SA and OA reduced ER stress and apoptosis, and increased the mitochondrial activity in cumulus cells (Marei *et al.,* 2017). In our study II, dietary supplementations with EFA, CLA and EFA+CLA could modify FA composition in the FF but were still ineffective in improving the oocyte developmental competence *in vitro*. However, the actual increased levels of ALA and *cis-9*, *trans-11* CLA as observed in the FF of animals within the different dietary groups provided us with a fairly accurate idea of physiological concentrations to be tested in our GC culture model for their effects on steroid production, marker gene expression and viability. These data were already discussed in section 2.2.2. Though, it remains to be shown whether manipulating dietary strategies in dairy cattle can achieve sufficient changes in PUFA levels to modulate oocyte competence.

In study II we speculate that adverse effects of EFA and CLA diets on oocyte competence could be associated with higher proportions of ALA and *cis-9, trans-11* CLA as observed in the FF. Data from previously discussed studies suggest that FF components might play a decisive role in determining oocyte competence, quality and ultimately overall fertility in bovine. However, in our study II, we did not see significant differences, which might be due to small experimental sample sizes. Another possible reason could be the experimental approach of oocyte retrieval from subordinate follicles. Since the ovaries were obtained from cows at different oestrous stages, it cannot be excluded that the developmental competence of the respective oocytes was occasionally suppressed by a dominant follicle. As a consequence, the sliced follicles from which oocytes were retrieved might not reflect the milieu of

preovulatory follicles. Further, dietary supplementation of EFA and CLA leading to increased proportions of ALA and *cis-9, trans-11* CLA in the FF might have adversely affected the developmental competence of oocytes within the follicles.

2.4 NEFAs and ovarian dysfunctions

In, the presented review (study III) several studies relating elevated levels of NEFAs to fertility issues in mammalian females including human and cattle are discussed. Severe and unregulated lipolysis is a hallmark of various metabolic diseases such as obesity, diabetes 2 and subclinical ketosis causing elevated level of NEFAs in plasma and FF of humans and ruminants. In humans and bovine, increased NEFA levels in the FF can alter the functionality of GCs, cumulus cells and oocytes. However, effects vary due to different chemical properties of fatty acids. The review discusses detrimental effects induced by SFAs in form of apoptosis in granulosa and cumulus cells subsequently affecting oocyte developmental competence in different species. From previous studies it is quite apparent that the metabolites of SFAs can induce apoptosis in human and bovine GCs (Mu *et al.,* 2001) (Vanholder *et al.,* 2005). However, as suggested by our data and by other previous studies it is evident that adequate E2 secretion by GCs might play a key role in combating the apoptotic effects of SFAs. SFAs impose detrimental effects on COCs by inducing ER stress and disrupting the mitochondrial function (Wu *et al.,* 2012). Unlike SFAs, effects of UFAs vary in different species. Interestingly, in human females OA is correlated with adverse pregnancy outcomes in obese women suffering from PCOS as the proportion of OA present in FF of obese PCOS women is comparatively higher as compared to obese non-PCOS women (Niu *et al.,* 2014). However, many studies have confirmed positive effects of UFAs, particularly of OA (Aardema *et al.,* 2011) and ALA (Marei *et al.,* 2017) in reducing the lipotoxicity induced by SFAs during oocyte maturation which might enhance blastocyst rates in bovine. Together, the review attempts to illustrate the modulatory effects of NEFAs on ovarian function during various metabolic states and their possible effects on overall health.

2.5 Hypoxia regulates steroidogenesis and differentiation of bovine GCs

Ovarian follicles undergo successive maturation, ovulation and luteinization followed by the formation of a functional CL which is either maintained or regressed depending upon fertilization of oocyte. In studies IV and V we studied the effects of the hypoxia i.e. O_2 deficiency that is increasingly prevalent through late follicular stages. Specifically, we studied how the low O_2 levels as found in preovulatory follicles affect the GCs and may contribute to early luteinization. Study V was then focused on specific effects of the key transcription factor during hypoxia, the HIF1 on steroidogenesis and cell proliferation. Both investigations were performed on primary GCs cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions using our well established estrogen active culture model of bovine primary GCs.

2.5.1 Low O_2 levels promote changes associated with early luteinization in GCs

It has been implied that large antral follicle of post-LH surge contains 1-2 % of O_2 levels. With mRNA microarray transcriptomic analysis we identified 1104 differentially expressed genes under hypoxia (1% O_2) compared to normoxia (21% O_2), indicating that O_2 levels can remarkably modulate gene expression profiles of bovine GCs. Importantly, the expression of *FSHR* and *LHCGR* were significantly down-regulated under hypoxia. This provides an indication that hypoxic conditions affects the FSH signaling in GCs. FSH signaling is essential for E2 production by inducing the expression of *CYP19A1* as also discussed earlier. And in fact, we observed a significant down-regulation of *CYP19A1* mRNA levels in GCs cultured at hypoxia. Contrary to FSH signaling, genes related to angiogenesis and endothelial cell migration was significantly up-regulated at hypoxia. Angiogenesis is a vital phenotypic feature of a fully functional CL (Reynolds *et al.,* 2000). Thus, it is obvious that up-regulation of genes involved in angiogenic processes complement luteinization in GCs. By using ingenuity pathway analysis (IPA), *HIF1A* which was expressed at relatively high levels, was identified as the chief upstream regulator of gene expression at hypoxia. HIF1transcription factor binds to the promoter regions of specific genes and induces vascularization in different tissues (Nishimura and Okuda, 2009). In line with this, IPA clearly revealed "*angiogenesis*" (p = 1.03E- 06) and "*vasculogenesis*" (p = 6.86E-07) as highly up-regulated biological functions at hypoxia. Apart from tissue vascularization, HIF1 also induces glucose metabolism as

observed in previous studies (Marin-Hernandez *et al.,* 2009). Likewise, expression of *GLUT1* and glucose transporter 3 (*GLUT 3*) were also stimulated at hypoxia. This observation is again consistent with an increased expression of *GLUT1* reported in early luteal cells in response to low O_2 level (3%) (Nishimura *et al.,* 2017). Thioredoxin interacting protein (TXNIP) is another critical regulator of glucose metabolism as reduced expression of *TXNIP* increases glucose uptake and also maintains glucose homeostasis in the liver cells (Chutkow *et al.,* 2008). This notion is also well in line with our study as *TXNIP* expression was considerably down-regulated thus affirming an increased glucose metabolism at hypoxia.

Hemoglobin alpha 1 (HBA1) gene was up-regulated in GCs cultured at hypoxic conditions. Increased levels of erythrocyte free HBA have been previously also reported in large antral follicles of hCG treated ovarian follicles (Thompson *et al.,* 2015). Thus, the presence of an up-regulated HBA gene in GCs cultured at hypoxia affirmed the assumption that hypoxia prevail in large antral follicles. Genes associated with inflammation and leucocyte migration are also widely induced in GCs of preovulatory follicles (Espey, 2006). The up-regulation of vanin 1 (*VNN1*), vanin 2 (*VNN2)*, prostaglandin E synthase (*PTGES*), complement C1q tumor necrosis factor-related protein 3 *(C1QTNF3)* and TNF alpha induced protein 3 *(TNFAIP3)* and TNF alpha induced protein 8 (*TNFAIP8*) in the present study suggest that early luteinization and ovulation processes might be supported in hypoxic conditions. Additionally, the unaltered expression of the prostaglandin-endoperoxide synthase 2 (*PTGS2)* gene, a marker of the post-LH response indicated that its regulation could be LH dependent and not controlled by hypoxia in preovulatory follicles. Further, we compared the present *in vitro* transcriptome with an *in vivo* post-LH surge staged GC transcriptome (Christenson *et al.,* 2013) for identifying the commonly regulated genes. This revealed that the regulation of 1007 genes at hypoxia is in accordance with bovine GCs isolated from the preovulatory LH surge follicles. Nonetheless, this strengthens our proposed hypotheses that hypoxia prevails in preovulatory follicles and induce early molecular events of differentiation in GCs towards luteinization in preparation of a functional CL. Surprisingly, P4 production was decreased in GCs cultured under hypoxia, possibly due to the absence of LH in the culture media. Physiologically, P4 synthesis is dependent on *LHCGR* and *HSD3B1* expression in GCs. In earlier *in vivo* studies, a similar down-regulation of the *LHCGR* and *HSD3B1* genes was observed *in vivo* post LH surge staged GCs, however, with P4 levels

remaining unaltered in early preovulatory ovarian follicles (Christenson *et al.,* 2013). Nevertheless, in both situations it remains to be explained how effects of the LH surge and of hypoxic conditions interact to regulate P4 production during the folliculo-luteal transition.

The transition of GCs to early luteal cells commences with a decreased cell proliferation (Stocco *et al.,* 2007). In previous studies, GCs that were isolated after the LH surge showed a steady decline in expression of cell proliferation markers, *CCND2* and *PCNA* (Nimz *et al.,* 2009). Our study revealed that GC proliferation in hypoxia is largely affected as indicated by pathway analysis using IPA. Canonical pathways like "estrogen-mediated S-phase entry," "cyclins and cell cycle regulation" and "cell cycle: G2/M DNA damage checkpoint regulation" were found to be significantly affected. Accordingly, cyclin dependent kinase inhibitor (CDKN) such as *CDKN1A*, *CDKN1B*, and *CDKN1C* were clearly up-regulated at hypoxia. Additionally, a zero order protein-protein interaction (PPI) network identified critical hub genes that were affected by hypoxia. Among these *ESR1* was found to be the most highly ranked hub gene with a degree of interaction = 70, followed by *KIAA0101* with a degree = 65. The high degree of these hub genes is inversely related to down-regulation of GC proliferation. ESR1 upon binding to estrogens undergoes nuclear translocation and binds to estrogen response element (ERE) thus regulating multiple target genes involved in cell cycle progression (Oviedo *et al.,* 2011). Similarly, *KIAA0101* is implied to functionally associate with *PCNA* (Emanuele *et al.,* 2011), another important gene of cell proliferation. Therefore, we speculate that down-regulation of *ESR1* and *KIAA0101* induced by hypoxia could further prevent GCs from entering into the S-phase of the cell cycle.

2.5.2 Steroidogenesis and proliferation is transcriptionally regulated by HIF1

In study V, we determined the transcriptional regulatory capacity of the HIF1 in cultured bovine primary GCs. In mammals including cows, IGF-1 and FSH induced steroidogenesis and cell proliferation is essential for ovarian follicle development. In this study, primary GCs were cultured with physiologically relevant concentrations of FSH (2, 10 or 20 ng) and IGF-1 (2, 25 or 50 ng). *HIF1A* mRNA expression was induced by 2 and 20 ng/ml of FSH, and 25 and 50 ng/ml of IGF1. Detection of HIF1A in the GC layer of differently staged ovarian follicles by immunohistochemistry suggested that HIF1 may play a role during folliculogenesis. Previously, pregnant

mare serum gonadotropins (PMSG) were also found to induce *HIF1A* expression in ovarian follicles of immature rats (Zhang *et al.,* 2015). This suggests that different physiological stimuli can increase HIF1 expression and activity in ovarian compartments.

In this study, we used chemical inhibition and gapmer mediated gene knockdown approaches to study HIF1 loss of function in GCs under both normoxic (21% O_2) and hypoxic condition (1% O_2). Echinomycin is a competitive inhibitor, which binds to the HRE and thus inhibits the transcriptional activity of HIF1(Kong *et al.,* 2005). In echinomycin treated GCs, the mRNA abundance of *HIF1A* was significantly down-regulated under both normoxic and hypoxic conditions. We speculate that suppression of HIF1 by echinomycin could trigger the expression of certain micro RNAs (miRNAs) such as miR-200, which could target the HIF1A transcripts in GCs. Under hypoxic conditions, HIF1A protein accumulates due to inhibition of the prolyl hydroxylase enzyme, which under normoxic conditions uses molecular O_2 to hydroxylate HIF1A thus targeting it for 26S proteasome dependent degradation (Chua *et al.,* 2010). The lower *HIF1A* mRNA expression levels under hypoxic conditions were surprising and need further elucidation. Possibly, *HIF1A* mRNA might be regulated by so far unknown feedback mechanisms leading to lower expression under hypoxic as compared to normoxic conditions in the used GC culture model.

Inhibition of HIF1 function lead to a down-regulation of VEGFA in cultured GCs at both, normoxic and hypoxic conditions. VEGFA is a known downstream target of HIF1A (Pagès and Pouysségur, 2005), and with its down-regulation, it became clear that HIF1 activity in GCs was suppressed upon echinomycin and HIF1 gapmer treatments. *VEGFA* mRNA expression of was also lower under normoxic as compared to hypoxic conditions. This was most probably due to the reduced stability of HIF1A proteins under normoxic conditions. VNN2 is known to be involved in inflammatory processes, such as leukocyte migration and adhesion. However, a gonadotropin dependent increase of VNN2 has been also reported in bovine GCs of preovulatory follicles prior to ovulation, thus suggesting its plausible physiological role during the ovulatory process in bovine (Sayasith *et al.,* 2013). Previously, in study IV, we already analyzed the expression of *VNN2* as it was up-regulated in GCs isolated from preovulatory follicles post-LH surge. Also, previously we observed that expression of *VNN2* was up-regulated by hypoxia in GCs. But, gapmer knockdown of

HIF1A lead to down-regulation of the *VNN2* mRNA both under normoxic and hypoxic conditions. This suggests that up-regulation of *VNN2* in GCs of the ovulatory follicle might be due to existing hypoxic conditions rather than induction by LH surge. Regulation of steroidogenic genes by HIF1 varies in different tissue as recorded previously. In mouse KK1 cells, (Kowalewski *et al.,* 2015) *STAR* is induced by HIF1, contrary to murine Ledyig cells (Wang *et al.,* 2019), where *STAR* is inhibited. While HIF1 in canine luteal cells inhibits *HSD3B* expression along with increased P4 production (de Carvalho Sousa *et al.,* 2016) and induces *HSD3B1* expression in murine leydig cells (Lysiak *et al.,* 2009). Correspondingly, HIF1 also induces *CYP19A1* expression in breast adipose stromal cells (Samarajeewa *et al.,* 2013) and inhibits the same in H295R steroidogenic adrenal cortical cells (Yu *et al.,* 2015). However, inhibition of HIF1 leads to a dose-dependent suppression in *CYP19A1* mRNA expression along with a reduced E2 production in bovine GCs under normoxic conditions.

Previously, in human breast adipose stromal cells, it was shown that HIF1 binds to the *CYP19A1* proximal promoter using chromatin immunoprecipitation (ChIP) analysis. This is in line with our finding as we also observed the binding of HIF1 to the *CYP19A1* proximal promoter. This suggests that *CYP19A1* could be a possible target for HIF1 under FSH and IGF-1 signaling conditions in GCs of developing follicles. However, under hypoxic conditions, knocking down HIF1A did not relay any effect on *CYP19A1* mRNA expression and E2 production. Hypoxic conditions favour HIF1 to induce synthesis of P4 by GCs during luteinization (Yoshioka *et al.,* 2014). Our results were in agreement with this observation as the inhibition of the HIF1 activity decreased the P4 production at hypoxia. This could be attributed to inhibition of *STAR* and *HSD3B* expression. Surprisingly, we observed an inconsistency between echinomycin and gapmer treatments toward *STAR* and *HSD3B1* mRNA expression. Under hypoxia, echinomycin significantly decreased the *STAR* and *HSD3B* mRNA expression, while both remain unaltered under normoxia. In contrast, HIF1A knockdown by gapmer consistently decreased *STAR* expression both under normoxic and hypoxic conditions while *HSD3B1* was induced at normoxia and remained unaltered at hypoxic conditions. However, decreased E2 and P4 levels reflect the changes at gene expression that were induced by gapmer gene knockdown.

The cell proliferation genes, *CCND2* and *PCNA* as also discussed earlier, have been used to identify the proliferation status of GCs. *PCNA* and *CCND2* mRNA levels were reduced under normoxia in the presence of echinomycin and gapmer, thus specifying the involvement of HIF1 in GCs proliferation. *PCNA* expression was reported to be down-regulated in rat GCs under normoxic conditions upon HIF1 inhibition (Zhang *et al.,* 2015). However, we observed up-regulation of *PCNA* gene expression at hypoxia following gapmer knockdown, though similar effects were not observed under echinomycin treatment. Nonetheless, echinomycin might cause non-specific inhibition as there is the possibility of off-target effects induced by pharmacological inhibitors (Miyazawa, 2011). Further, flow cytometry data confirmed that HIF1 knockdown could induce cell cycle progression under hypoxia as observed in *PCNA* gene expression. Based on gapmer and flow cytometry data we infer that HIF1 can conversely regulate cellular proliferation under normoxic or hypoxic conditions. Having reported such effect the study showed that cell proliferation by HIF1 is differentially regulated at normoxic and hypoxic conditions. These data could increase our understanding of how HIF1 activity, upon IGF-1 and FSH stimuli in ovarian compartment can modulate its effector targets differentially. Overall, study V provided molecular evidence regarding the transcriptional regulation of steroidogenesis by HIF1 in GCs under either normoxic or hypoxic conditions.

2.6 Conclusion and outlook

Higher levels of NEFAs in the FF of postpartum dairy cows can affect GC function and ultimately can restrict ovulation as well. Previously, higher NEFA concentrations have been already associated with poor fertility outcome in form of inactive ovaries, reduced oocyte developmental competence and even altered embryonic transcriptomes. Based on our *in vitro* and *in vivo* data we could affirm that exposure of higher concentration of FFAs particularly of UFAs to follicular GCs significantly hampered their ability of estradiol biosynthesis and ovulation. This inferred that higher levels of UFAs in FF might be active participants impairing steroidogenesis in ovarian GCs. This reflection could provide new approaches towards designing therapeutic strategies that are highly needed to improve postpartum fertility. Counteracting the effects imposed by NEFAs with pharmacological inhibitors might provide promising approaches in recovering the negative impacts of elevated NEFAs as observed in obese women and high lactating cows. In this context, several studies

have confirmed significant improvements in blastocyst development following treatment with certain stress inhibitors *in vitro*. Also, more studies emphasizing on intracellular signaling, especially PKA, PIK3/PKB and mitogen-activated protein kinases (MAPK) pathways could aid in better understanding of interaction between fatty acids and reproductive hormones FSH and IGF-1, which could help to generate novel therapeutics for treating subfertility in dairy cows. Besides focusing on GCs, oocyte and embryonic development, metabolic alterations in dairy cows can be functionally adjusted through dietary intervention with lipids, which can help establishing a more positive energy balance state during postpartum periods and generate better quality oocytes for embryo production and implantation. Fatty acid composition of the diet could also alter the FF composition as evident in study II by higher proportion of ALA and *cis-9 trans-11* CLA in FF. Effects of such higher proportion of these fatty acids on subsequent oocyte developmental potential is also apprehended. Nevertheless, a conclusive affirmation on the beneficial effects of dietary lipid supplementation in dairy rations on improving the oocyte competence still awaits further investigations. Additionally, despite of prevailing *in vivo* hypoxic conditions in preovulatory follicles final differentiation and luteinization of GCs is adequately maintained and regulation of steroidogenesis by the HIF1transcription factor in response to hypoxic conditions is progressively carried out by GCs. These findings might be instrumental in revealing the role of *in vivo* restricted O_2 levels in promoting differentiation and luteinization of somatic follicular cells.

All in all, comprehensive measures are required to reduce the prevalence of metabolic stress induced by NEFAs resulting in undesired reproductive outcomes in high yielding dairy cows. In this context, manipulation of animal feeding strategies during different timelines of pregnancy and lactation might prove effective in keeping the energy balance decent and promote desired reproductive outcomes.

3. Individual studies

Study I.

Elevated free fatty acids affect bovine granulosa cell function: a molecular cue for compromised reproduction during negative energy balance

Sharma A, Baddela V.S, Becker F, Dannenberger D, Viergutz T, Vanselow J. *Endocrine connections* (2019), 8(5): 493–505. (doi:10.1530/EC-19-0011)

Brief summary:

The study aimed at investigating the effects of increased physiological concentrations of PA, SA and OA on steroidogenesis, proliferation and viability of bovine GCs. Both PA and SA significantly up-regulated the expression of *CD36*, *FSHR, LHCGR, CYP19A1, HSD3B1, CCND2* and increased the E2 production, while OA down-regulated the expression of these genes except *CD36* up-regulation and reduced E2 production. Interestingly, *STAR* was down-regulated by all fatty acids and combination treatment. E2 was significantly reduced after combination treatment. *In vitro* effects of OA were validated *in vivo* by injecting BSA conjugated OA or non-conjugated BSA into the largest, presumably dominant follicles (10-19mm). The E2 concentrations were reduced significantly in the FF recovered 48 h post OA injection. Also, in recovered antral GCs the expression of *CD36* was significantly up- and that of *CYP19A1* and *STAR* was significantly down-regulated. The ovulation rates of OA injected follicles tended to be reduced. Overall, the study indicated that elevated free fatty acid concentrations, especially of OA can potentially target functional key genes in GCs both *in vitro* and *in vivo*.

Endocrine CONNECTIONS | A Sharma *et al.* | Effects of free fatty acids on granulosa cells | 8:5 | 493–505

RESEARCH

Elevated free fatty acids affect bovine granulosa cell function: a molecular cue for compromised reproduction during negative energy balance

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Abstract

High-yielding dairy cows postpartum face the challenge of negative energy balance leading to elevated free fatty acids levels in the serum and follicular fluid thus affecting the ovarian function. Here, we investigated effects of physiological concentrations of palmitic acid (PA), stearic acid (SA) and oleic acid (OA) on the viability, steroid production and gene expression in a bovine granulosa cell (GC) culture model. Treatment with individual and combined fatty acids increased the *CD36* gene expression, while no significant apoptotic effects were observed. Both PA and SA significantly upregulated the expression of *FSHR*, *LHCGR*, *CYP19A1*, *HSD3B1*, *CCND2* and increased 17β-estradiol (E2) production, while OA downregulated the expression of these genes and reduced E2. Interestingly, *STAR* was equally downregulated by all fatty acids and combination treatment. E2 was significantly reduced after combination treatment. To validate the effects of OA, *in vivo* growing dominant follicles (10–19 mm) were injected with bovine serum albumin (BSA) with/without conjugated OA. The follicular fluid was recovered 48 h post injection. As in our *in vitro* model, OA significantly reduced intrafollicular E2 concentrations. In addition, expression of *CD36* was significantly up- and that of *CYP19A1* and *STAR* significantly downregulated in antral GC recovered from aspirated follicles. The ovulation rates of OA-injected follicles tended to be reduced. Our results indicate that elevated free fatty acid concentrations specifically target functional key genes in GC both *in vitro* and *in vivo*. Suggestively, this could be a possible mechanism through which elevated free fatty acids affect folliculogenesis in dairy cows postpartum.

Key Words

- palmitic acid
- stearic acid
- oleic acid
- estradiol
- progesterone

Endocrine Connections (2019) **8**, 493–505

Introduction

High-yielding dairy cows suffer post-parturition from ovarian dysfunctions such as prolonged calving intervals and delayed cyclic resumption due to various factors (1). One of such risk factor is negative energy balance (NEB). Catabolism and mobilization of stored triglycerides lead to increased systemic concentrations of non-esterified fatty acids (NEFA) like palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1) and of β-hydroxybutyric acid (BHBA) (2). Such excessive lipid mobilization has been associated with metabolic and reproductive disorders (3) affecting both the cumulus oocyte complex morphology and embryo viability (4). Elevated plasma levels of PA, SA, OA and of linoleic acid have been shown in dairy cows postpartum as well as their infiltration into the follicular fluid (5). Since granulosa cells (GC) are the most important somatic constituents

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of the ovarian follicle regulating follicle development along with the production of follicular fluid and plasma estradiol (6). Elevated NEFA levels have detrimental effects on bovine GC (7). In human GC, saturated fatty acids (PA and SA) are known to induce apoptosis, causing potential reproductive abnormalities such as amenorrhea, which is often observed in obese women (8). However, on the contrary, OA (monounsaturated fatty acid) supplementation has shown to protect exocrine pancreatic cells from PA-induced apoptosis thus reducing the rate of pancreatic diseases caused by obesity (9). Also, OA co-supplementation diminishes pro-inflammatory and cytotoxic effects of SA accumulation in human aortic endothelial cells (10). *In vitro* exposure of COCs to PA and SA induces endoplasmic reticular stress, mitochondrial damage and increased apoptosis in cumulus cells thus impairing oocyte developmental competence (11), while OA is harmless even at high concentrations (12, 13, 14). However, studying the effects of these major NEFAs, particularly of PA, SA and OA both individually and in combination on steroidogenesis, cell proliferation and apoptosis in an *in vitro* bovine GC culture model might contribute to our understanding of the collective effects of elevated free fatty acid concentrations during NEB on bovine reproduction. Thus, in the present study, we examined the effects of these fatty acids on GC functionality at elevated, but physiological concentrations as observed in follicular fluid during NEB. For solubilization, fatty acids were conjugated with bovine serum albumin (BSA) to mimic the physiological conditions as close as possible. Specifically, we studied the individual and combined effect of PA, SA and OA on proliferation, apoptosis and steroidogenesis in our well-established serum-free, estrogen-producing GC culture model and validated effects of OA in a bovine *in vivo* animal model, which allows to test the effects of individual fatty acids on the differentiation of individual follicles while largely avoiding superimposed systemic influences.

Materials and methods

In vitro primary GC culture

Primary GC culture was performed as previously described (15). For testing the effects of fatty acids, the media (supplemented α-MEM) were replaced after 48 h with the same media containing different concentrations of PA (50, 100 and 200 µM), SA (50, 100, 200 µM) or OA (100, 200 and 400 µM) as BSA conjugates or BSA as vehicle control (fatty acids and BSA from Sigma-Aldrich). For BSA conjugation, fatty acids were dissolved in 1–2 mL chloroform/methanol solution (v/v = 2/3) and evaporated to dryness under nitrogen atmosphere. Subsequently, a solution of fatty acid-free BSA (w/v = 10% in PBS) was prepared and added. The BSA/fatty acid mixtures were then vortexed, sonicated and subjected to a mild heat treatment (<40°C) until a clear solution was formed. Subsequently, BSA/fatty acid solutions were sterilized by filtration (0.02 µm) and kept at −20°C until addition to the cell culture media (v/v = 1/10). All control wells received the same volume of BSA as the test groups with highest fatty acid concentrations.

Steroid hormone estimation

Concentrations of 17β-estradiol (E2) and progesterone (P4) in the conditioned media were determined by competitive 3H radioimmunoassay (RIA) with rabbit-raised antibodies purified by affinity chromatography as described earlier (16).

Cell viability and cell cycle analysis

Attached GCs were thoroughly washed twice with 1× PBS and trypsinized using 250 µL TryplE solution (Thermo Fisher) followed by incubation (37°C, 20 min). After centrifugation the cells were merged with non-attached cells from spent media. All cells were again centrifuged, washed with 1 mL MEM and analyzed for apoptosis using an Annexin V FITC kit (Miltenyi Biotec). Cell pellets were re-suspended in 100 µL of 1× binding buffer to which 10 µL of Annexin V reagent was added. After gentle pipetting, the tubes were incubated in dark for 15 min followed by washing and resuspension in 500 µL 1× binding buffer. Then, 5 µL of PI (propidium iodide, 500 µg/mL) were added to the cells and gently mixed just prior to flow cytometric analysis. The fluorescence signal was quantified from single cells (10,000 counts) by a flow cytometer (Gallios, Beckman-Coulter) and the data obtained were analyzed using the Kaluza software (Beckman-Coulter).

For cell cycle analysis, the combined (attached and non-attached) cells were washed and dissolved in 300 µL of 1× PBS. Then, 70% ice cold ethanol was added dropwise into the cell suspensions and stored (−20°C, 2 h). Later, cells were pelleted (300 ***g***, 10 min, 4°C), re-suspended in 1 mL RNase solution (1 mg/mL) and incubated (37°C, 5% CO_2, 30 min). The PI reagent was added to the cells and further incubated in the dark (37°C, 30 min). Lastly, the fluorescence signal was quantified from single

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cells (10,000 counts) by a flow cytometer (EPICS-XL, Beckman-Coulter). Data were analyzed using the MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

RNA isolation and cDNA synthesis

Total RNA preparation was done using innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol and quantified using NanoDrop1000 Spectrophotometer (Thermo Scientific). Later, cDNA was prepared using SensiFAST cDNASynthesis Kit (Bioline, Luckenwalde, Germany) from 200 ng RNA as previously described (17).

Quantitative real-time PCR (qPCR) analysis

qPCR was performed using SensiFAST SYBR No-ROX (Bioline, London, UK) with gene-specific primers (Table 1) in a Light Cycler 96 instrument (Roche) as described previously (18). The abundance of transcripts was normalized relative to *RPLPO* as a suitable reference gene.

Animals, follicle injection and aspiration

Animal experiments were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (Aktenzeichen: 7221.3-1.1-068/17). Fourteen Holstein heifers aged 13–14 months, weighing 380–400 kg were kept indoors at our institute and fed a standard diet. Ultrasonographic follicular monitoring (transportable equipment with 5 MHz linear probe, Hitachi CS 9100 Picker) was carried out aside from the normal heat detection to verify the cycle stage. Special attention was given to the development of larger follicles about 2–3 days before intrafollicular injection to recognize growing and regressing follicles within the follicular population. An adapted amount of BSA without/with 400 µM conjugated OA was injected into the selected growing dominant follicle (10–19 mm). A sector scanner with a 6.5 MHz puncture transducer and a special designed carrier (VETEC GmbH, Rostock, Germany) to guide a needle with an outer diameter of 0.5 mm was used. Epidural anesthesia was induced by 5 mL of lidocaine-hydrochloride injectable-2% (Procamidor, WdT, Garbsen, Germany). To monitor effects on the ovulation of injected follicles 14 animal were further monitored for cycling activity. Sizes of the injected dominant follicles were measured and sample collection was done using transvaginal ultrasonographically guided follicle aspiration 48 h after injection. Recovered follicular fluid was immediately stored on ice (<15 min) and taken to the laboratory. Further, the follicular fluid was centrifuged for 3 min at 15,000 ***g*** to obtain clear follicular fluid and GC pellets. The GC pellets were instantly frozen in liquid nitrogen followed by storage at −80°C for gene expression analysis while the obtained follicular fluid was stored at −20°C for hormone estimation.

Lipid analysis and fatty acid analysis

Follicular fluid samples of approximately 200 µL were dropwise added to 8 mL chloroform/methanol (2:1, v/v) at room temperature. The solution contained C19:0 as an internal standard. The general sample preparation procedure is as earlier described (19). The fatty acid analysis of the follicular fluid was performed using capillary gas chromatography with a CP-Sil 88 CB column (100 m × 0.25 mm, Agilent) that was installed in

Table 1 List of primers used for gene quantification by q-PCR.

Gene	Sequence	Size (bp)	Accession no.
CD36	For: GCTCCTTAAGCCATTCTTGGAT Rev: CACCAGTGTCAACGCACTTT	151	NM_001278621.1
CYP19A1	For. GCTTTTGGAAGTGCTGAACCCAAGG Rev: GGGCCCAATTCCCAGAAAGTAGCTG	172	NM_174305
FSHR	For: TCACCAAGCTTCGAGTCATCCCAAA Rev: TCTGGAAGGCATCAGGGTCGATGTA	189	NM_174061
CCND2	For: CGCAGGGCCGTGCCGGACGCCAAC Rev: CACGGCCCCCAGCAGCTGCAGATGG	279	NM_001076372
PCNA	For: GTGAACCTGCAGAGCATGGACTCGT Rev: CGTGTCCGCGTTATCTTCAGCTCTT	192	NM_001034494
LHCGR	For: GCATCCACAAGCTTCCAGATGTTACGA Rev: GGGAAATCAGCGTTGTCCCATTGA	205	NM_174381
HSD3B1	For: TGTTGGTGGAGGAGAAGGATCTG Rev: GCATTCCTGACGTCAATGACAGAG	208	NM_174343
RPLP0	For: TGGTTACCCAACCGTCGCATCTGTA Rev: CACAAAGGCAGATGGATCAGCCAAG	142	NM_001012682

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a PerkinElmer gas chromatograph CLARUS 680 with a flame ionization detector and split injection (PerkinElmer Instruments). The detailed gas chromatography conditions were set as described (20).

Statistical analysis

All experiments were carried out in triplicates with different GC preparations. Data of tested groups were analyzed using one-way ANOVA (all pair wise multiple comparison procedures with Holm-Sidak method) or unpaired *t* test using the Sigma Plot 11.0 Statistical Analysis System (Jandel Scientific, San Rafael, CA, USA). Differences were considered significant at $P<0.05$.

Results

Effects of individual fatty acids on GC functionality *in vitro*

Cell morphology

As evident from the light microscopic photographs, treatment with increased PA, SA and OA concentrations resulted in morphological changes. GC changed their appearance from a regular fibroblast-like appearance under BSA vehicle control treatment to an increasingly round-shaped morphology with increased accumulation of intracellular vesicles at highest PA, SA or OA concentrations (Fig. 1).

Steroid hormone concentrations

Estimations of P4 and E2 concentrations within conditioned media after the 8-day culture period revealed that PA and SA significantly stimulated E2 production as compared to the BSA control, whereas OA strongly reduced E2 concentrations. In contrast, levels of P4 remained unaltered after PA and SA treatment, but were significantly reduced only at highest OA concentration (Fig. 2).

Gene expression analysis

qPCR data showed that transcript abundance of the cell cycle regulator *CCND2* was significantly up-regulated by PA and SA at high concentrations. In contrast, OA significantly reduced the *CCND2* transcripts at all concentrations. Transcript abundance of the proliferation marker *PCNA* however, was only affected by PA showing significant upregulation at the highest concentration (Fig. 3). Interestingly, we also found differential, in part opposing regulation of other functional key genes. Whereas the transcript abundance of the fatty acid transporter *CD36* was strongly upregulated by all three fatty acids (Fig. 4), key transcripts of estradiol and progesterone production, *CYP19A1* and *HSD3B1* as well as those of gonadotropin receptors, *FSHR* and *LHCGR*, showed increased upregulation after PA (Fig. 5A) and SA treatment (Fig. 5B), but clearly reduced expression in OA-treated cells (Fig. 6). In contrast, all fatty acids reduced transcript abundance of *STAR*, the rate-limiting factor of steroidogenesis (Fig. 7).

Cell viability and proliferation

Flow cytometry analysis of Annexin V-FITC/PI-stained cells showed no statistically significant differences in the proportions of apoptotic cells after treatment with highest concentrations of PA, SA or OA (200 µM, 200 µM and 400 µM, respectively) as compared to vehicle control. However, the percentage of apoptotic cells tended to be higher in the PA and SA treated groups,

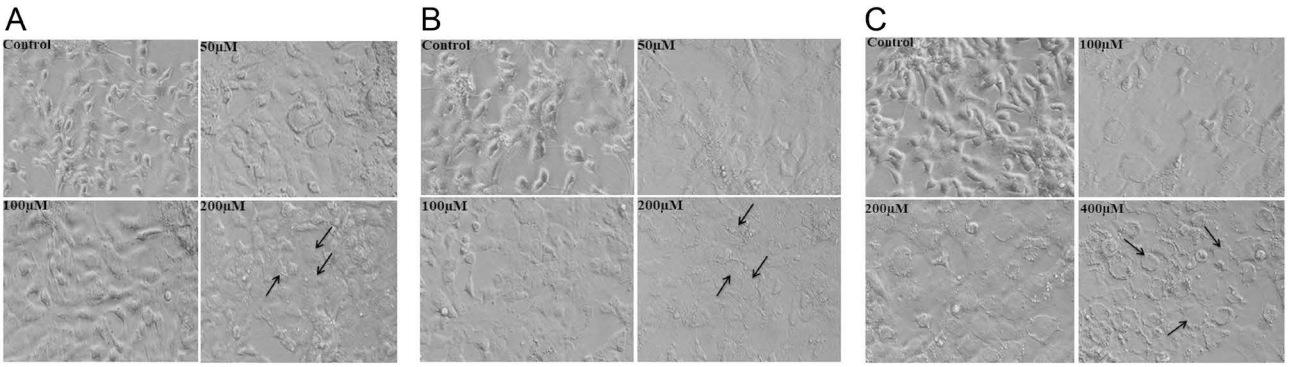

Figure 1
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on *in vitro*-cultured GC morphology. Photomicrographs were obtained from Nikon TMS-F inverted microscope. Arrows indicate lipid droplet formation in the cells.

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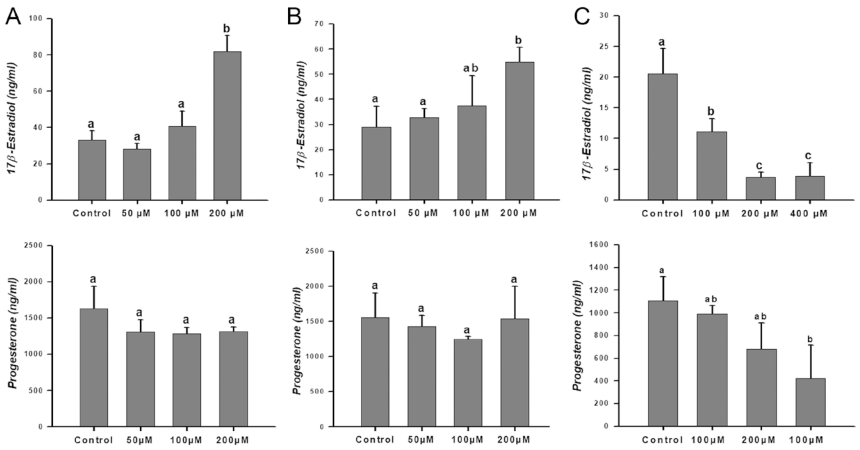

Figure 2
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on steroid hormone concentrations conditioned media of cultured bovine GC. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

but lower with OA (Table 2). The proportion of viable cells was virtually unchanged in all treatment groups, whereas the number of dead cells was significantly higher in OA group. Further, none of the fatty acids induced a significant shift of cell cycle phases in cultured GC. Flow cytometric analysis revealed similar proportions of cells in SubG0/G1, G0/G1, S and G2/M phases (Table 3).

Effects of fatty acid combinations

To test combined effects of PA, SA and OA, highest effective concentrations of these fatty acids (200 µM, 200 µM and 400 µM) were mixed and added to cultured GC. As in case of individual fatty acids the morphology of the cells dramatically changed as compared to BSA vehicle controls (Fig. 8A). The results also revealed that co-supplementation had no influence on P4 production, but significantly reduced E2 levels (Fig. 8B). qPCR quantification of key transcripts showed no significant regulation of *CYP19A1*, *HSD3B1*, *FSHR*, *LHCGR CCND2* and *PCNA*, but highly upregulated *CD36* transcripts and significantly downregulated *STAR* (Fig. 9). Results from flow cytometry by Annexin V-FITC/PI staining revealed that the percentage of apoptotic cells was unchanged, whereas that of viable cells was significantly reduced by the combined fatty acid treatment. In particular, the proportion of dead cells was remarkably increased by the combined treatment as compared to the BSA controls (Table 4). However, we did not find significant difference in cell cycle stages (Table 5).

Effect of intrafollicular OA injection on GC functionality *in vivo*

To validate the data obtained from cultured GC, we tested effects of OA in an animal model by directly injecting growing dominant follicles. This approach will expose antral GCs directly to increased OA concentrations while largely avoiding systemic effects. In some animals, post-injection follicle differentiation and ovulation rate was also monitored, whereas in other animals injected follicles were aspirated 48 h later in order to collect follicular fluid and cells therein for hormone production and gene expression analysis.

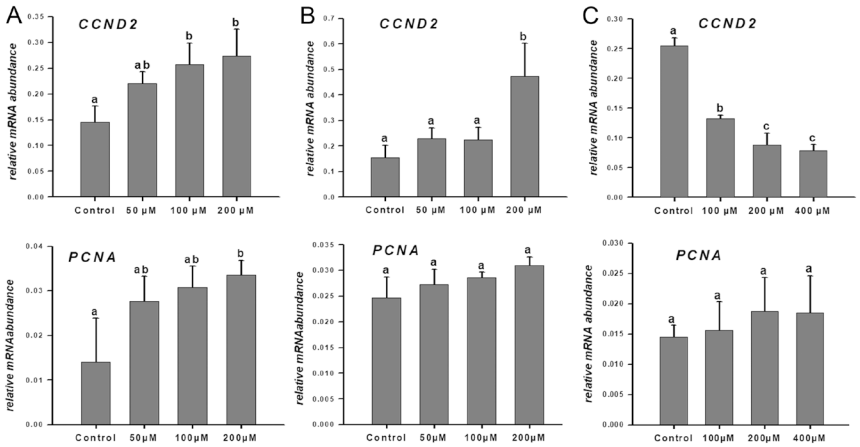

Figure 3
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on transcript abundance of the cell proliferation marker genes *CCND2* and *PCNA*. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm-Sidak test: $n = 3$, $P < 0.05$.).

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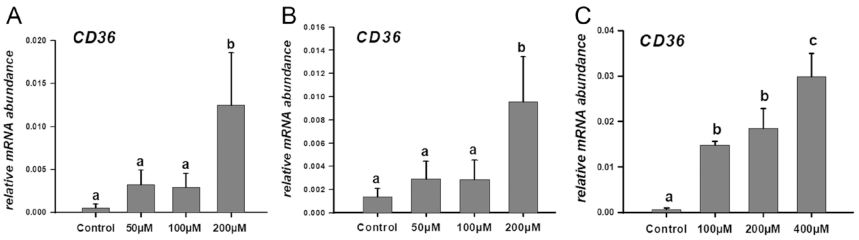

Figure 4
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on transcript abundance of the fatty acid translocase/*CD36* gene. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

Ovulation rates

From 14 injected growing dominant follicles (one per animal) that were kept for monitoring the ovulatory competence, nine were injected with BSA, out of which seven follicles advanced to the ovulatory stage (77%), while only one out of five follicles injected with OA ovulated (20%) within 96 h post injection. Although not significant yet the ovulatory competence tended to be reduced following the OA as compared to the BSA injections.

Fatty acid analysis by gas chromatography

Gas chromatography analysis of follicular fluid samples obtained 48 h post intrafollicular injections (BSA with/without conjugated OA) revealed varying proportions of different fatty acids. However, the percentage of OA (46.51 ± 5.8%) in OA-injected follicles was significantly higher as compare to BSA (13.59 ± 2.5%) injected follicles (Supplementary Table 1, see section on supplementary data given at the end of this article).

Steroid hormone estimation

Follicular fluid obtained from aspirated follicles was subjected to RIA for estimating the concentration of P4 and E2, respectively. The results revealed that intrafollicular OA injection remarkably reduced the production of E2 compare to the vehicle control (BSA), while P4 level remained largely unaffected (Fig. 10A).

Gene expression analysis

qPCR analysis showed significantly higher levels of *CD36* and lower levels of *CYP19A1* and *STAR* transcripts in GC recovered after intrafollicular OA injection. However, the transcript level of *CCND2* remained unaltered throughout (Fig. 10B).

Discussion

Considerable changes in the serum metabolite profile of post-partum dairy cows are approximately mirrored in the follicular fluid of dominant follicles to which pre-ovulatory

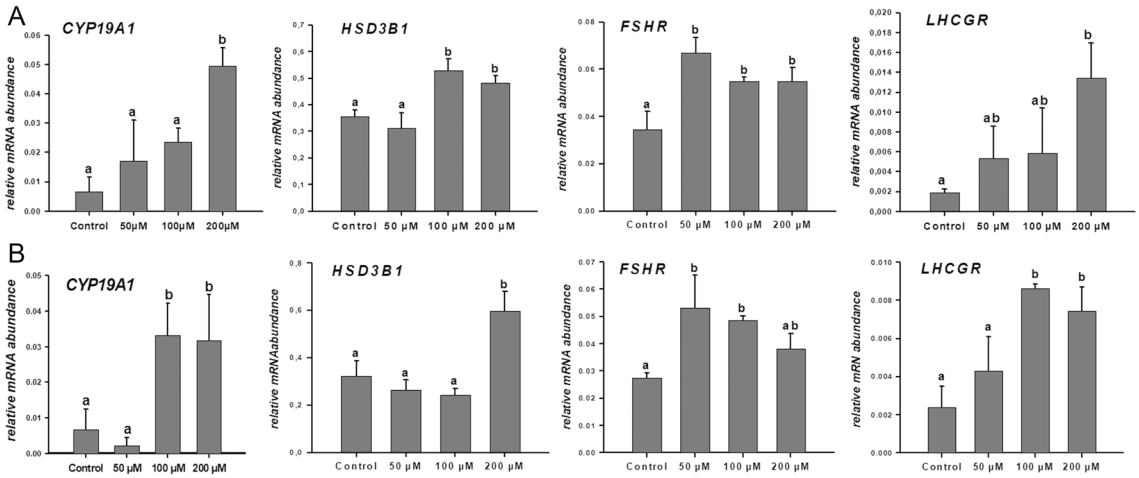

Figure 5
Effects of increasing concentrations of (A) palmitic acid (C16:0) and (B) stearic acid (C18:0) on key transcripts of estradiol and progesterone production (*CYP19A1*, *HSD3B1*) and gonadotrophin receptors (*FSHR*, *LHCGR*). Transcript abundance was normalized to the reference gene *RPLPO.* The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

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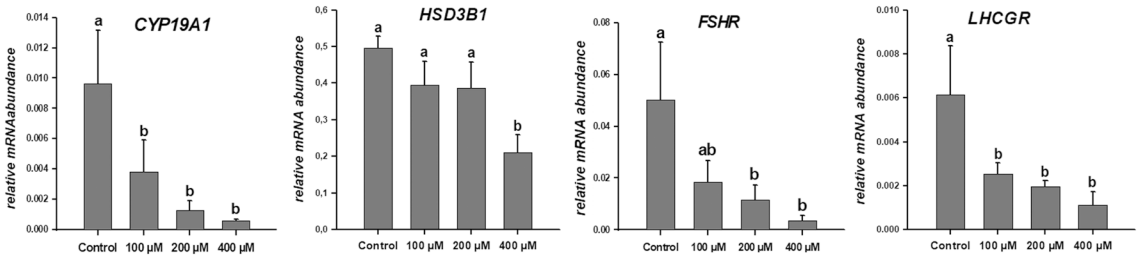

Figure 6
Effects of increasing concentrations of oleic acid (C18:1*cis*-9) on key transcripts of estradiol and progesterone production (*CYP19A1*, *HSD3B1*) and gonadotrophin receptors (*FSHR*, *LHCGR*). Transcript was abundance normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

oocytes and GC are exposed (21). Thus, to determine if elevated intrafollicular concentrations of free fatty acids affect GC functionality and thus may contribute to declined fertility rates as experienced by cows during NEB, in the present study, we analyzed the *in vitro* effects of major free fatty acids both separately and in combination on GC functionality. The tested concentrations of PA, SA and OA were based on earlier *in vivo* experiments which provided estimated physiological concentrations of free fatty acids in the follicular fluid of short-term fasted cows (22). Additionally, we knew from earlier reports that concentrations of OA is highest in the follicular fluid of cows and the present data showed that GC functionality is adversely modulated *in vitro*; thus, we specifically selected OA for intrafollicular injection to validate whether the effects of OA observed in our GC culture model can be transferred to the *in vivo* situation. In contrast to our previous studies (23) and to those of others (11), where the fatty acids had been solubilized by using ethanol, we solubilized the fatty acids by conjugation with BSA as previously published (24). On one hand, this certainly mirrors the physiological situation in the follicular fluid more closely, and on the other hand, it enabled us to reproducibly solubilize not only the monounsaturated fatty acid OA, but also the saturated fatty acids PA and SA. According to our previous (unpublished) observations, the effects of ethanol solubilized PA or SA, but not those of OA, on cultured GC showed extreme variability. Very likely, this might be due to different physicochemical properties of saturated vs unsaturated fatty acids. In any case, this let us eventually change our experimental procedure. However, it is also obvious from our present data that the effects of OA on GC were very similar independent of the method of solubilization.

PA, SA and OA alter cell morphology of cultured bovine GC by inducing lipid accumulation

In vitro supplementation of fatty acids separately and in combination induced significant alterations in the morphology and physiological activity of cultured GC. Their morphology closely resembled the morphology of foam cells as observed in OA-treated smooth muscle cells and macrophages (25, 26, 27). In addition, these morphological changes were associated with a highly induced expression of fatty acid translocase *CD36* (FAT) known to mediate fatty acid uptake (28). This clearly suggests that all three fatty acids induce their own uptake by the cells. Our results are quite consistent with other studies in primary bovine mammary epithelial cells where mRNA abundance of *CD36* was markedly increased when the cells were cultured with PA, SA, OA, linoleate or an conjugated linoleic acid isomer (*trans*-10, *cis*-12 18:2) (29, 30) and in rat type II pneumocytes, where FAT/CD36 is expressed mediating the uptake of PA (31). This is well in line with data of the present study showing that PA, SA and OA induce massive accumulation of intracellular vesicles previously (23) identified as lipid droplets in cultured bovine GC.

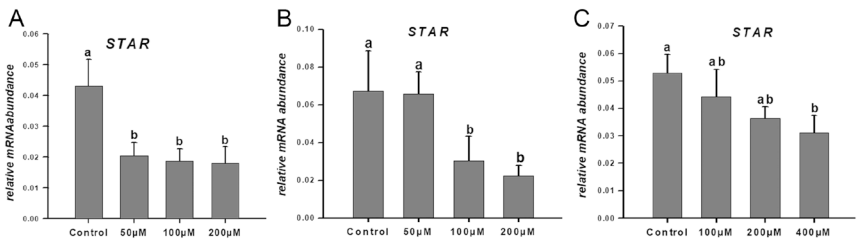

Figure 7
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on gene expression of *STAR*. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

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Table 2 Cell viability was determined by Annexin V-FITC staining assay as described in the Materials and methods section.

Tested groups	Parameters		
	Viable cells (%)	Apoptotic cells (%)	Dead cells (%)
(I) Control	79.27 ± 1.2	13.72 ± 2.4	5.9 ± 1.2[a]
(II) Palmitic acid (200 µM)	74 ± 2.9	19.2 ± 3	5.8 ± 0.2[a]
(III) Stearic acid (200 µM)	76.9 ± 4.2	15.8 ± 2.6	6% ± 1.9[a]
(IV) Oleic acid (400 µM)	67.8 ± 3.5	11.3 ± 0.8	20.4 ± 3.6[b]

The data are shown as means ± S.E.M. (Holm–Sidak test: $n = 3$). Significant differences are indicated by letters a and b ($P < 0.05$).

In vitro cell viability and proliferation

In spite of dramatic morphological alteration of cultured GC under PA and SA supplementation, even at their highest concentrations these fatty acids did not elicit significant apoptotic effects. As mentioned earlier saturated fatty acids, particularly PA and SA, induce apoptosis in human GC (8) and lipoapoptosis in human β-cells, while the unsaturated fatty acids OA and linoleate have no such effects (32). But it is equally important to consider the fact that in cows, follicles that secrete higher E2 levels are selected for continued growth up to the ovulatory stage (33, 34) and higher E2 level is known to increase both GC proliferation and follicle cell differentiation (35, 36, 37). A positive correlation between elevated E2 levels and GC survival has been reported, where higher E2 levels not only protected GC from Fas ligand-induced apoptosis, but also increased the percentage of cells entering from G1 to S phase of the cell cycle, in addition to an increased cyclin D2 protein expression (38). In the present study, both PA and SA stimulated the E2 production significantly along with upregulation of the *CCND2* gene. This could effectively mediate the protective effect of high estradiol levels in PA- and SA-treated GC despite of their apoptotic impacts in other mammalian cells as described earlier. As per OA, there were no signs of apoptosis in cultured GC even at its highest tested concentration. OA has been previously reported to have no apoptotic effects on CHO cells and β-islet cells even at high concentrations. This is in line with the observation that OA causes lipid accumulation more effectively as compared to saturated fatty acids such as PA (39). The cells ability to effectively incorporate fatty acids into cytoplasmic triglycerides (TG) might serve as a protection against their pro-apoptotic effects. OA is more readily incorporated into triglyceride and is therefore considered less apoptotic as compared to saturated fatty acids (40), which are poor substrates for TG synthesis. In addition, OA is known to even mediate protection against PA-induced apoptosis (41). In response to FSH, *CCND2* mRNA expression rapidly increased in GC (42, 43), while FSH β-null mutant mice display decreased *CCND2* mRNA levels (44). This is well in line with our observation that PA and SA increased the FSHR transcript levels, and as a consequence probably increased the responsiveness of the GC toward this gonadotropin, which in turn may have indirectly induced elevated *CCND2* transcript abundance. In contrast, OA steadily decreased the *FSHR* expression, thus leading to reduced FSH responsiveness. The observed steep downregulation of *CCND2* transcript abundance might be therefore a consequence of reduced FSH signaling under OA treatment. Both observations thus are indicative for the important role of FSH signaling for GC viability and proliferation.

Our results showed that combined PA, SA and OA treatment did also not induce apoptotic effects and more interestingly, significantly lowered the E2 production, thus suggesting that OA effects seem to dominate those of PA and SA in the mixture. This could be partially due to the observation that OA effectively rescues saturated fatty acid-induced apoptosis under the activity of the enzyme stearoyl-CoA desaturase (SCD1), catalyzing the conversion of saturated fatty acid into monounsaturated fatty acids, thus directing the saturated fats into TG synthesis and away from pathways leading to apoptosis.

Steroidogenesis is modulated differentially *in vitro* by saturated and monounsaturated fatty acid

To determine the physiological effects of PA, SA and OA on GC we looked upon the transcriptional activity of key

Table 3 Cell proliferation was determined by flow cytometer analysis.

Tested groups	Cell cycle phase			
	SubG0/G1 (%)	G0/G1 (%)	S Phase (%)	G2/M (%)
(I) Control	13.23 ± 6.4	91.2 ± 1.5	6.8 ± 1.2	1.9 ± 0.25
(II) PA 200 µM	10.16 ± 3.2	88.6 ± 1.0	8.1 ± 1.4	3.2 ± 0.4
(III) SA 200 µM	24.3 ± 9	89.2 ± 1.3	6.8 ± 0.4	3.8 ± 1
(IV) OA 400 µM	11.9 ± 4.07	92 ± 2.9	4.1 ± 1.7	3.8 ± 1.25

PA, SA, OA represent palmitic acid, stearic acid and oleic acid, respectively. The data are shown as means ± S.E.M., Holm–Sidak test, $n = 3$).

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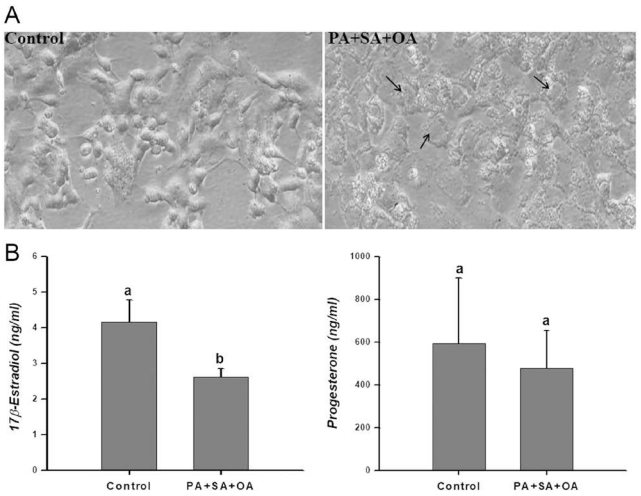

Figure 8
(A) Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on *in vitro* cultured GC. Photomicrographs were obtained from Nikon TMS-F inverted microscope. Arrows indicate lipid droplet formation in the cells. (B) Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on the steroid hormone concentration in conditioned media of cultured bovine GC. The data are shown as means ± SEM. Different letters indicate significantly different means (unpaired *t* test; $n = 3$, $P < 0.05$).

genes involved in gonadotropin signaling such as *FSHR* and *LHCGR* and in steroidogenesis such as *CYP19A1*, *STAR* and *HSD3B1*. Since both folliculogenesis and steroidogenesis are closely dependent on the coordinated actions of FSH and LH with their receptors on GC and thecal cells of ovarian follicles (45). *CYP19A1* expression has been found to be elevated by SA supplementation in murine GC (46). This is in line with our data showing that both PA and SA increased the expression of *FSHR*, *LHCGR*, *CYP19A1* and *HSD3B1*, and thus, stimulated E2 production. In contrast, all these genes and E2 production were significantly downregulated by OA. Interestingly, the expression of *STAR* was significantly downregulated by all three fatty acids and by the combined treatment. However, only OA and the combined treatment also resulted in a significantly reduced E2 production, whereas E2 levels were increased by PA and SA treatment. Most likely, in case of OA treatment E2 reduction could be largely due to the reduced transcription of *HSD3B1* and *CYP19A1*, whereas higher levels of E2 in PA and SA treated GC can be attributed to upregulation of *CYP19A1* (47). Nevertheless, a clear effect of reduced transcription of *STAR* that is considered as the rate-limiting factor of steroid hormone synthesis (48) might be obscured by the presence of androstenedione that is a preferred substrate for *CYP19A1* to produce E2 in GC (49) in our cell culture medium.

The presented results on OA effects are in accordance with our previous study (23), but are contradictory to data reported by others (7). These authors concluded that OA despite of inhibiting proliferation stimulates the production of E2 in cultured bovine GC. However, our results on stimulatory effects of PA and SA on E2 production are in line with the same study and also with other reports showing that exposure of

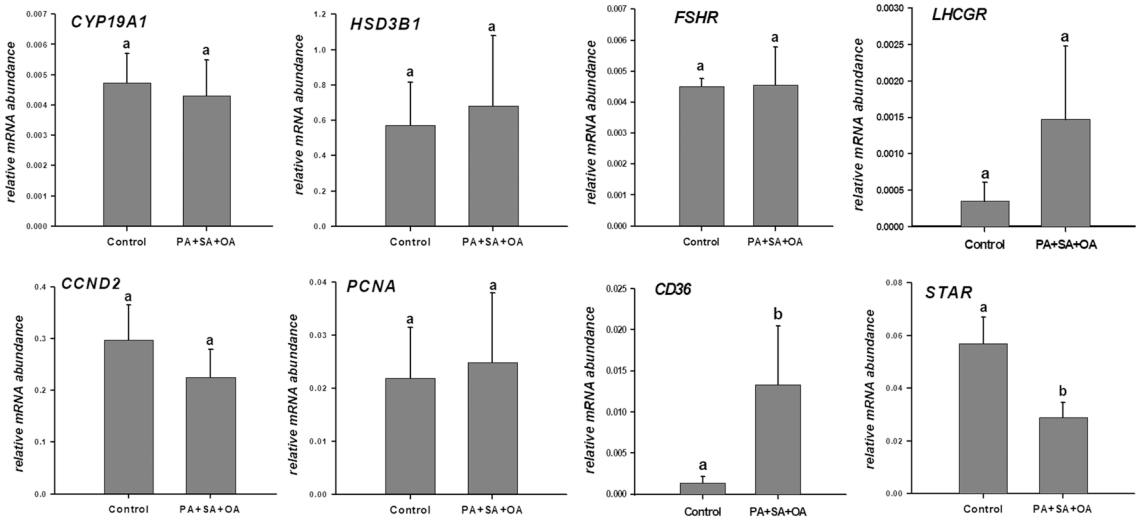

Figure 9
Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on the abundance of key transcripts of estradiol, progesterone production (*CYP19A1*, *HSD3B1*), gonadotrophin receptor genes (*FSHR*, *LHCGR*), cell proliferation markers (*CCND2*, *PCNA*), *CD36* and *STAR*. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (unpaired *t* test: $n = 3$, $P < 0.05$).

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Table 4 Cell viability was determined by Annexin V-FITC staining assay as described in the Materials and methods section.

Tested groups	Parameters		
	Viable cell (%)	Apoptotic cells (%)	Dead cells (%)
(I) Control	55.7 ± 9.5[a]	34 ± 8.3	9 ± 0.9[a]
(II) PA + SA + OA	29.7 ± 1.09[b]	32.3 ± 3.2	36.9 ± 4.1[b]

Palmitic acid (PA, 200 µM), stearic acid (SA, 200 µM) and oleic acid (OA, 400 µM) were co-supplemented to cultured GC and were compared to control. The data are shown as means ± S.E.M., unpaired *t* test: ($n = 3$). Significant difference is indicated by letters a and b ($P < 0.05$).

androgen-producing cells to saturated fatty acids significantly increased androgen production (50). In this context, it is important to point out that PA and SA clearly increase the expression of gonadotropin receptors *FSHR* and *LHCGR*, whereas OA showed opposite effects. FSH is known to induce the expression of *LHCGR* via protein kinase A (PKA) and phosphoinositide3-kinase (PI3K) pathways in rat GC (51). *LHCGR* is highly expressed in GC during the pre-ovulatory stage to enable responsiveness to the LH surge thus leading to ovulation, oocyte maturation and corpus luteum formation (52). The downregulation of both *FSHR* and *LHCGR* might therefore affect the FSH signaling pathway leading to compromised GC functionality due to elevated NEFAs specifically by OA. Both PA and SA upregulated *CYP19A1* expression with subsequent increase of the E2 production, while OA downregulated the *CYP19A1* expression while reducing E2 production. These data, but in particular, our results on the effects of combined fatty acids (PA+SA+OA) may give some hints to better understand the *in vivo* situation. *STAR* expression is downregulated by all three fatty acids as well as by the combined treatment. This suggests that elevated *in vivo* concentrations of fatty acids during NEB might cause cumulative effects on *STAR* expression in steroidogenic cells similar to our *in vitro* tested combination. However, it has to be considered as well that about 10–15% of steroid biosynthesis is known to occur via *STAR* independent mechanisms (53, 54) as some other proteins like MLN4 have been known to possess *STAR*-like activity in promoting cholesterol flux into mitochondria (55). From our data, it became very clear that the saturated fatty acids tested (PA, SA) elicited very different, partly opposing effects in GC as compared with effects of OA as a monounsaturated fatty acid.

Intrafollicular OA injection modulates GC function *in vivo* and impedes ovulation

To validate the *in vitro* data obtained from our GC culture model we used an *in vivo* approach by directly injecting the antral cavity of growing dominant follicles. This approach will expose antral GC directly to the injected agent while largely avoiding superimposing systemic effects. *In vivo* effects of Insulin-like Growth Factor 1 (IGF-1) has been also studied by intrafollicular injection to reveal the mechanisms underlying follicle deviation in heifers and mares (56). However, to our knowledge no functional *in vivo* study on the role of fatty acids on GC function and hormone concentration by direct injection of follicles in heifers has been carried out so far. Our *in vivo* data clearly provided an affirmation of the results obtained *in vitro*. As discussed above *in vitro* data indicated that OA has a dominant effect as compared to other fatty acids *in vitro*, thus, we specifically selected this fatty acid for intrafollicular injection. Fatty acid analysis of follicular fluid samples by gas chromatography revealed significantly higher proportions of OA in OA injected as compared to BSA injected follicles even 48 h post-injection, but also demonstrated that proportions of most of the other fatty acids was not markedly altered.

The results are consistent with those reported *in vitro*, with a reduced E2 concentration detected in follicular fluid recovered from OA injected follicles. Additionally, only 22% of OA injected follicles reached the ovulatory stage as compared to 77% of BSA injected follicles achieving ovulation. This indicates a tendency of OA to inhibit ovulation. Since, dominant follicles secrets sufficient E2 to induce the pre-ovulatory LH surge for successful ovulation (57), reduced E2 concentrations as observed in

Table 5 Cell proliferation was determined by flow cytometer analysis.

Tested groups	Cell cycle phase			
	SubG0/G1 (%)	G0/G1 (%)	S phase (%)	G2/M (%)
(I) Control	13.36 ± 3.7	94.8 ± 0.5	3.8 ± 0.8	1.5 ± 0.2
(II) PA + SA + OA	14 ± 3.1	94.5 ± 0.5	3.9 ± 0.8	1.75 ± 0.45

Palmitic acid (PA, 200 µM), stearic acid (SA, 200 µM) and oleic acid (OA, 400 µM) were co-supplemented to cultured GC and were compared to control. The data are shown as means ± S.E.M., unpaired *t* test: $n = 3$.

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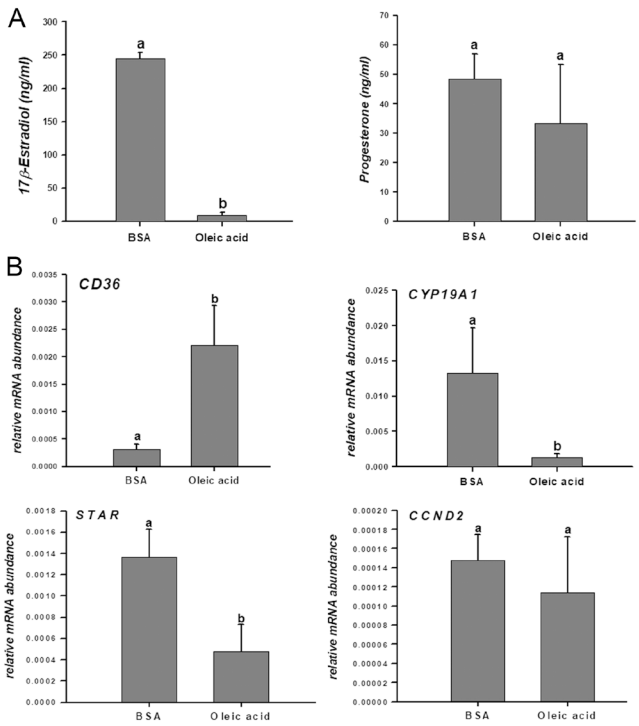

Figure 10
(A) Effects of intrafollicular oleic acid (C18:1*cis*-9) injection on the steroid hormone concentration in follicular fluid recovered 48 h post injection. The data are shown as means ± S.E.M. Different letters indicate significantly different means (unpaired *t* test; $n = 3$, $P < 0.05$). (B) Effects of intrafollicular oleic acid (C18:1*cis*-9) injection on transcript abundance of *CD36*, *CYP19A1*, *STAR* and *CCND2*. BSA and OA (400 µM) indicate vehicle control and tested fatty acid respectively. Transcript abundance was normalized to the reference gene *RPLPO.* The data are shown as means ± S.E.M. Different letters indicate significantly different means (unpaired *t* test; $n = 3$, $P < 0.05$).

the follicular fluid recovered from OA injected follicles might be responsible for the observed lower ovulation rate. A similar decrement of E2 concentrations by OA was also observed in the *in vitro* experiments both in independent as well as in combination treatments. Also, further investigation revealed that gene expression of *CD36* was significantly up-regulated while both *CYP19A1* and *STAR* were significantly down-regulated in GC recovered from OA injected follicles. These results evidently indicate that following intrafollicular injection, OA was actively transported into the GC, with further down-regulation of both *CYP19A1* and *STAR*. Since *CYP19A1* in bovine is the key enzyme for E2 synthesis, the resulting lower concentration of E2 in follicular fluid is clearly in line with suppressed *CYP19A1* expression. Thus, considering the *in vivo* data we anticipate that OA effects might dominate among the mixed fatty acids that are mobilized during the period of NEB in postpartum dairy cattle.

Conclusion

In the present study, we showed that PA and SA in contrast to OA induced the expression of key genes of steroidogenesis and E2 production. These differential effects might be attributed to differential saturation or stereo metric positions of double bonds in fatty acids. Combined effects of fatty acids are largely dominated by mostly negative effect of OA on both hormone production and transcript abundance. Suggestively, this might mirror the *in vivo* situation, where OA is the most up-regulated fatty acid during NEB in the follicular fluid. Additionally, lower *CYP19A1* and *STAR* expression followed by lower E2 production after intrafollicular OA injection confirms the assertion that this fatty acid impedes steroidogenesis both *in vitro* and *in vivo* in bovine GC. Collectively, these effects of NEFA might therefore be responsible for mechanism through which the NEB influences the reproductive performance of high-yielding dairy cows during the early postpartum period. Finally, we suggest that these findings may partly be also applicable to obese women with increased serum concentrations of free fatty acids suffering from impaired fertility.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/EC-19-0011.

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

Funding
The project is funded by Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program.

Acknowledgements
Authors appreciate Veronika Schreiter, Maren Anders, Swanhild Rodewald and Christian Plinski, for their technical support throughout the experiments. They also thank the Argrar-Milchhof-Stiewe GbR in 18279 Lalendorf, Germany for providing the animals and supporting our study.

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Received in final form 10 January 2019
Accepted 29 March 2019
Accepted Preprint published online 29 March 2019

Study II.

Effects of dietary fatty acids on bovine oocyte competence and granulosa cells

Sharma A, Baddela V.S, Roettgen V, Vernunft A, Viergutz T, Dannenberger D, Hammon H.M, Schoen J, Vanselow J. *Frontiers in Endocrinology* (2020), 11:87. (doi: 10.3389/fendo.2020.00087)

Brief summary:

The aim of the study was to assess the effects of dietary EFAs on the developmental competence of oocytes in cows and on follicular GCs function. Lactating German Holstein cows were supplemented with different lipids from week 9 antepartum until week 8 postpartum in four groups (i) CTRL (coconut oil), (ii) EFA: linseed and safflower oil (iii) CLA: Lutalin®, and (iv) EFA+CLA: mixture of linseed oil, safflower oil and Lutalin®. For *in vitro* embryo production, cows at week 9 postpartum were slaughtered to obtain the ovaries and oocytes were collected by the slicing method. EFA, CLA or EFA+CLA diet groups had no effects on overall oocyte development competence as compare to the CTRL group. Fatty acid analysis by gas chromatography revealed higher proportions of ALA and *cis-9*, *trans-11* CLA in the FF. Further, we explicitly tested the effects of different concentrations of ALA and *cis-9, trans-11* CLA on cultured bovine GCs. Upon supplementation, both fatty acids were actively transported into the cultured GCs as indicated by altered morphology and up-regulation of *CD36* gene expression. Also, ALA and *cis-9*, *trans-11* CLA significantly down-regulated the expression of *STAR, CYP19A1, FSHR, LHCGR* and decreased the E2 production. Thus, dietary EFAs could not alter *in vitro* oocyte development competence. However, higher proportions of ALA and *cis-9*, *trans-11* CLA in the FF might affect GC functionality, which might restrict follicular development and ovarian cyclicity in high yielding cows.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 25 February 2020
doi: 10.3389/fendo.2020.00087

Effects of Dietary Fatty Acids on Bovine Oocyte Competence and Granulosa Cells

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Here we assessed the effects of dietary essential fatty acids on the developmental competence of oocytes in cows and on the functionality of follicular granulosa cells (GC). Lactating German Holstein cows were supplemented from week 9 ante partum (ap) until week 8 post-partum (pp) in four dietary groups designed as (i) control (CTRL: coconut oil), (ii) essential fatty acid (EFA: linseed and safflower oil), (iii) conjugated linoleic acid (CLA: Lutalin®), and (iv) EFA+CLA (mixture of linseed oil, safflower oil and Lutalin®). EFA, CLA or EFA+CLA supplementation did not improve *in vitro* embryo production. However, higher proportions of α-linolenic acid (ALA) and *cis-9*, *trans-11* CLA were observed in the follicular fluid suggesting the exposure of GC to relatively high levels of ALA and *cis-9, trans-11* CLA. Consequently, we tested different concentrations of ALA and *cis-9, trans-11* CLA in a bovine GC culture model for their effects on steroid production, marker gene expression and viability. Both fatty acids upregulated *CD36* and downregulated the expression of *FOXL2*, while ALA significantly increased *SOX 9* transcript levels. Both ALA and *cis-9, trans-11* CLA reduced the *CCND2* expression and *cis-9, trans-11* CLA induced apoptosis. ALA and *cis-9*, *trans-11* CLA significantly down-regulated the expression of *STAR, CYP19A1, FSHR, LHCGR* and decreased the 17β-Estradiol (E2) and progesterone (P4) production. In conclusion, dietary lipids did not improve *in vitro* embryo production, while ALA and *cis-9*, *trans-11* CLA affected the morphology and functionality of GC. This could suggestively lead to compromised follicle development and ovarian cyclicity in dairy cows.

Keywords: alpha-linolenic acid, conjugated linoleic acid, estradiol, granulosa cells, gene expression

OPEN ACCESS

Edited by:
Artur Mayerhofer, Ludwig Maximilian University of Munich, Germany

Reviewed by:
Qi Yu, Peking Union Medical College Hospital (CAMS), China
Winnie Shum, ShanghaiTech University, China

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *26 November 2019*
Accepted: *11 February 2020*
Published: *25 February 2020*

Citation:
Sharma A, Baddela VS, Roettgen V, Vernunft A, Viergutz T, Dannenberger D, Hammon HM, Schoen J and Vanselow J (2020) Effects of Dietary Fatty Acids on Bovine Oocyte Competence and Granulosa Cells. Front. Endocrinol. 11:87. doi: 10.3389/fendo.2020.00087

INTRODUCTION

Dietary supplements can improve the reproductive outcome in cows by increasing the energy intake thus reducing the extent of negative energy balance (NEB) experienced postpartum (1). Polyunsaturated fatty acids (PUFA) enriched diets in cattle are known to alleviate NEB during early lactation (2). PUFA at the cellular level maintain several functions such as membrane stability, regulation of transcription factors, cell proliferation and differentiation (3). Among PUFA, essential fatty acids such as linoleic acid (LA) and α-linolenic acid (ALA) are not synthesized in cows, human and pigs and must be supplied through the diet (4). *In vivo*, the nutritional effects of PUFA are well-known to affect the oocyte metabolism and early development in livestock species (5). The fatty acid (FA) profile of the follicular fluid (FF) is well-correlated to the type of dietary FA surrounding the

oocytes as dietary fats can alter the FA composition in cumulus and granulosa cells (GC) thus influencing oocyte quality (6). Ewes fed with PUFA diets showed higher proportions of LA and docosahexaenoic acid (DHA) in FF and cumulus cells with lower proportions in oocytes (7). The size of dominant follicles is reported to increase in cows fed with PUFA diets as compared to cows fed with monounsaturated fatty acids (MUFA) (8). Increased follicle size is supposed to improve both oocyte quality and corpus luteum (CL) function in cows (9). Larger ovulatory follicles with lower rates of pregnancy losses were found in cows fed with flaxseed (9.8%) compared to those fed with sunflower seed (27.3%) (10). Among other dietary fats, conjugated linoleic acid (CLA) isomers (*cis-9*, *trans-11* and *trans-10*, *cis-12*) comprise a group of PUFA derived from LA during incomplete biohydrogenation by the ruminal flora in cattle and sheep (11). CLA increases the plasma concentration of insulin-like growth factor-1 (IGF-1) in cows, thus promoting conception rates (12). CLA supplements decrease the milk fat excretion during the early lactation period saving energy in order to combat the physiological NEB (13). As CLA at physiological concentrations has been found to affect nuclear maturation of cumulus oocyte complexes (COCs) and similarly, LA could also negatively affect both oocyte and embryo development due to altered glutathione peroxidase and superoxide dismutase mRNA expression (14). Negligible effects of ALA and LA diets on cleavage and embryo development from oocytes collected *via* ovum pick up (OPU) have been reported (15). Cows fed with omega-3 (ω-3) enriched diet failed to enhance the ovulation even upon ovarian stimulation (16). Dietary effects of essential fatty acids particularly ALA and CLA or their mixture on oocyte developmental competence as well as their effects on GC functionality still remain to be elucidated in detail. Thus, the primary objective of the study was to determine the effect of essential fatty acids especially of ALA and CLA by abomasal supplementation in lactating cows to assess the effects on *in vitro* preimplantation embryo development. We also analyzed the FA compositions of FF in the different dietary groups and elucidated the effects of increased concentrations of ALA and *cis-9*, *trans-11*CLA on the morphology, hormone production, viability, and gene expression of cultured bovine GC.

MATERIALS AND METHODS

Animals and Dietary Supplementation

Handling of animals and the experimental design were approved by the federal state of Mecklenburg Western-Pommerania, Germany (LALLF M-V TSD 7221.3-1-038/15). Forty German Holstein-Friesian cows at 18th week of gestation in their 2nd lactation were kept in a free-stall barn at the Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. Cows included in the study were surgically fitted with rumen fistulas and abomasal tubes to bypass rumen derived biohydrogenation of supplemented FA (17). The cows were studied in 5 blocks each consisting of 8 cows (2 cows per supplementation group and per block) from week 9 ante partum (ap) up to week 8 post-partum (pp) in their 3rd lactation. The ingredients and the composition of the diet, separately for lactation and dry-off diet are shown in **Supplementary Table 1**. FA composition of all four oil supplements is shown in **Supplementary Table 2**. Cows were supplemented daily from week 9 ap until week 8 pp either with 76 g/d coconut oil (CTRL $n = 9$), 78 g/d linseed and 4 g/d safflower oil (EFA $n = 9$) providing a ω-6/ ω-3 FA ratio of 1:3, Lutalin® (CLA $n = 10$, 10 g/d of *cis-9, trans-11,* and *cis-10, trans-12* CLA isomer, respectively) or a mixture of linseed and safflower oil plus Lutalin® (EFA + CLA $n = 10$). Following last dietary supplementation, cows in wk 9 pp were slaughtered for oocyte collection by the slicing method. In between, due to abortions in week 6 ap, 4 cows had to be removed from the study though two of these cows were replaced. Thus, altogether 38 cows were examined throughout the experimental duration.

FA Analysis by Gas Chromatography

FF from large follicles (10–24 mm) was aspirated from ovaries of the experimental cows after slaughter. Approximately 500 μL of FF was dropwise added to 8 mL chloroform/methanol (2:1, v/v) at room temperature. The fatty acid C19:0 used as an internal standard. The procedure for sample preparation was performed as described earlier (18). The fatty acid analysis of the FF was executed using capillary gas chromatography with a CP-Sil 88 CB column (100 m × 0.25 mm, Chrompack-Varian, Lake Forest, CA, USA) which was installed in a PerkinElmer gas chromatograph Autosys XL with a flame ionization detector (PerkinElmer Instruments, Shelton, CT, USA). The complete gas chromatography conditions were as described earlier (19). Hydrogen at a flow rate of 1 mL/min was used as the carrier gas while the split ratio was 1:20 with the injector and detector set to 260 and 280°C, respectively.

In vitro Production (IVP) of Embryos

The IVP protocol was executed using the IVF Bioscience media suite formulated for bovine embryo production according to the manufacturer's instructions (IVF Bioscience, Falmouth, United Kingdom, catalog # 61002, 61004, 61003, 61010, 61001, and 62000) (20). Briefly, 6–10 COCs obtained from cows of different diet groups after slaughter were matured in BO-IVM media and incubated for 24 h (38.5°C, 6% CO_2). For IVF, 2 × 10^6 sperms/ml from proven fertile Holstein Friesian bull were added to BO-IVF media containing matured oocytes and incubated for 18–22 h (38.8°C, 6% CO_2, 21% O_2). Putative zygotes were cultured in BO-IVC media and overlaid by BO-Oil and incubated at 38.8°C, 6% CO_2, 6% O_2, 88% N_2. Embryo cleavage was examined 48 h post IVF and subsequent embryo development was evaluated on day 8 post IVF. To count total cell number (TCN), embryos were fixed in 4% (v/v) paraformaldehyde (Sigma 252549) and stored at 4°C overnight. Embryos were mounted on glass slides and stained with Hoechst 33258 (Sigma B1155). Images were captured by confocal laser scanning microscope LSM 800 assembled with ZEN software (Carl Zeiss, Oberkochen, Germany).

Primary GC Culture

Primary GC culture and bovine serum albumin (BSA)-FA conjugate preparations were done as described earlier (21). For testing the effects of actual follicular concentration

of ALA (Sigma-Aldrich,L2376) and *cis-9*, *trans-11*CLA (Sigma-Aldrich,16413) the media were replaced every 48 h with supplemented α-MEM media containing different concentrations of ALA (20, 40, and 80 μM) or *cis-9*, *trans-11* CLA (15, 30, and 60 μM) as BSA conjugates or non-conjugated BSA as vehicle control (Sigma-Aldrich A7030). All control wells received the same volume of BSA as the test groups with highest fatty acid concentration. The conditioned media collected on the 8th day of culture were stored at −20°C for steroid hormone estimation, while the remaining cells were lysed for RNA isolation.

Steroid Hormone Analysis

Concentration of E2 and P4 of GC conditioned media were determined by competitive 3H–radioimmunoassay (RIA) with rabbit-raised antibodies purified by affinity chromatography as performed earlier (22). Radioactivity was measured in a liquid scintillation counter (LSC) with an integrated RIA-calculation programme (TriCarb 2900 TR; PerkinElmer).

Flow Cytometry

On day 8 of GC culture, cells were thoroughly washed twice with phosphate buffered saline (PBS) and then trypsinized using 250 μl TryplE solution (Thermo Fischer, USA) at 37°C for 20 min. Post trypsinization, all detached cells were centrifuged and resuspended in 1 ml MEM and analyzed for viability and apoptosis using an Annexin-V FITC/PI kit (Miltenyi biotec, Germany). Cells were centrifuged and pellets were re-suspended in 100 μl of binding buffer to which 10 μl of Annexin V reagent was added and kept for incubation in the dark for 15 min. followed by washing and resuspension in 500 μl binding buffer. Next, 5 μl of PI (Propidium iodide, 500 μg/ml) was added to the cells with gently mixing just prior to flow cytometric analysis. The fluorescence signal was quantified from single cells (10,000 counts) by a flow cytometer (Gallios, Beckman-Coulter, Germany) and the data obtained was analyzed using the Kaluza-software (Beckman-Coulter, Germany).

RNA Isolation, cDNA Synthesis, and Real Time-Quantitative PCR (RT-qPCR)

RNA isolation was performed using the innuPREP RNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol and quantified with NanoDrop1000 Spectrophotometer (Thermo Scientific, Bonn, Germany). Later, cDNA was synthesized using the SensiFAST cDNASynthesis Kit (Bioline, Luckenwalde, Germany) from 200 ng RNA as done previously (23). The RT-qPCR was executed for gene expression analysis, using SensiFAST SYBR No-ROX (Bioline) with gene-specific primers (**Supplementary Table 3**) in a Light Cycler 96 instrument (Roche, Mannheim, Germany) (24).

Statistical Analysis

IVF data were analyzed by one-way ANOVA (Holm-Sidak method, all pair wise multiple comparison procedure). The mean proportions of follicular fatty acids in supplemented diet groups was compared by one way ANOVA (Holm-Sidak method) or ANOVA on ranks (Dunn's Method) for pairwise multiple comparisons. The RIA, RT-qPCR gene expression and flow cytometry data (at least three biological replicates) were analyzed by one-way repeated measure ANOVA (all pair wise multiple comparison or multiple comparison vs. control procedures with Holm-Sidak or Dunnett test where applicable) or ANOVA on ranks when normality test failed using SigmaPlot 11.0. Significant changes were recognized if $P < 0.05$.

RESULTS

FA Analysis of Dietary Lipid Supplements in Follicular Fluid

The proportions of different FA were analyzed in the FF of all four dietary groups (**Table 1**). The percentage of ALA was significantly ($P < 0.05$) higher in the EFA and EFA + CLA diet groups with 10.19 ± 2.36 % and 12.56 ± 2.02%, respectively, as compared to the CTRL (2.81 ± 0.46 %) and CLA diet groups (2.44 ± 0.45 %). While *cis-9*, *trans-11* CLA isomer was significantly higher ($P < 0.05$) in the CLA and EFA+CLA diet groups with 0.4 ± 0.12% and 0.29 ± 0.03%, as compared to the CTRL and EFA diet groups (0.15 ± 0.15% and 0.07 ± 0.03%). Further, due to higher proportions of ALA and *cis*-9, *trans*-11 CLA in FF, we speculate that GC are exposed to relatively high levels of ALA and *cis*-9, *trans*-11 CLA which might affect the functionality of GC residing within. Consequently, we tested different concentrations of both ALA and *cis-9*, *trans-11* CLA in our GC culture model.

In vitro Embryo Development

A total number of 260 oocytes were recovered after slicing the ovaries obtained from cows of each diet group. We did not supplement the *in vitro* culture media with any FA for IVP. As for IVP we purely analyzed the developmental competence of obtained oocytes *in vitro*. The percentage of oocytes which underwent cleavage 48 h after fertilization and the blastocyst rates at the 8th day are presented in **Table 2**. The cleavage rates of oocytes recovered from the EFA and CLA diet groups tended to be higher (51.7% ± 9.3% and 53.3% ± 5.1%) as compared to those of EFA + CLA (36.2% ± 10.6%) and of the CTRL (34 ± 8.1%), however without statistical significance. Similarly, the rate of blastocysts generated from the cleaved embryos in the CTRL (23.5% ± 11.7%) and in the EFA, CLA and EFA+CLA diet groups (17.4% ± 7.9%, 26.1 ± 11.2%, and 15.2% ± 8.4%, respectively) remained statistically indifferent. Blastocysts generated on day 8 post IVF from each diet groups were stained with Hoechst 33258 to assess the TCN (**Figure 1**). The TCN count in EFA, CLA and EFA + CLA were 92.1 ± 10.1, 124.9 ± 18.9, and 121 ± 13.2, respectively. as compared to TCN count of the CTRL (71.2 ± 12.1).

Effect of ALA and *cis-9*, *trans-11* CLA on GC Functions *in vitro*

Steroid Hormone Production

Concentrations of E2 and P4 were determined by RIA in the conditioned media of *in vitro* cultured GC treated with different concentrations of ALA and *cis-9*, *trans-11* CLA (**Figure 2**). ALA significantly reduced ($P < 0.05$) the E2 production at all the

TABLE 1 | Fatty acid profile of follicular fluid analyzed by Gas chromatography.

Fatty acids	CTRL	CLA	EFA	EFA+CLA
Saturated fatty acids (SFA)				
C10:0	0.24 ± 0.1	0.23 ± 0.21	0.28 ± 0.22	0.26 ± 0.23
C11:0	0.01 ± 0.005	0.009 ± 0.003	0.02 ± 0.03	0.01 ± 0.006
C12:0	0.33 ± 0.11	0.27 ± 0.07	0.31 ± 0.11	0.26 ± 0.1
C13:0	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
C14:0	1.1 ± 0.19^{ab}	1.26 ± 0.19^{a}	1 ± 0.16^{b}	1.14 ± 0.3^{ab}
C15:0	0.63 ± 0.12	0.73 ± 0.12	0.72 ± 0.16	0.73 ± 0.18
C16:0	10.95 ± 0.66	11.38 ± 1.09	12.89 ± 2.19	11.42 ± 2.1
C17:0	0.86 ± 0.24	0.84 ± 0.17	0.87 ± 0.23	0.93 ± 0.23
C18:0	11.21 ± 1.1	11.62 ± 1.08	10.66 ± 1.03	10.51 ± 1.2
C20:0	0.18 ± 0.07	0.22 ± 0.05	0.25 ± 0.05	0.23 ± 0.08
C21:0	0.13 ± 0.09^{a}	0.23 ± 0.11^{b}	0.14 ± 0.03^{ab}	0.26 ± 0.08^{ab}
C22:0	0.45 ± 0.14	0.56 ± 0.12	0.59 ± 0.16	0.58 ± 0.21
C23:0	0.21 ± 0.06	0.33 ± 0.16	0.73 ± 0.91	0.33 ± 0.23
C24:0	0.84 ± 0.31	0.98 ± 0.27	1.09 ± 0.26	1.01 ± 0.3
Sum SFA	27.22 ± 1.15	28.8 ± 1.68	29.65 ± 3.62	27.75 ± 3.98
Monounsaturated fatty acids (MUFA)				
C14:1*cis*-9	0.16 ± 0.27^{a}	0.02 ± 0.01^{ab}	0.02 ± 0.01^{b}	0.07 ± 0.16^{b}
C16:1*cis*-9	1.66 ± 0.3^{a}	1.38 ± 0.27^{a}	1.29 ± 0.14^{a}	0.93 ± 0.09^{b}
C17:1*cis*-9	0.43 ± 0.05^{a}	0.32 ± 0.27^{ab}	0.17 ± 0.1^{b}	0.14 ± 0.17^{b}
C18:1*cis*-9	8.78 ± 1.43^{a}	7.67 ± 1.63^{a}	8.2 ± 3.46^{a}	5.15 ± 1.8^{b}
C18:1*cis*-11	0.9 ± 0.16^{a}	0.78 ± 0.17^{ab}	0.89 ± 0.36^{ab}	0.6 ± 0.11^{b}
C18:1*trans*-9	0.11 ± 0.05^{a}	0.08 ± 0.04^{b}	0.06 ± 0.01^{b}	0.07 ± 0.01^{ab}
C18:1*trans*-11	0.5 ± 0.37	0.36 ± 0.08	0.32 ± 0.07	0.44 ± 0.2
C20:1*cis*-11	0.04 ± 0.01	0.04 ± 0.01	0.07 ± 0.07	0.03 ± 0.01
Sum MUFA	12.75 ± 1.5^{a}	10.88 ± 2.06^{a}	11.18 ± 3.92^{a}	7.51 ± 1.92^{b}
Polyunsaturated fatty acids (PUFA)				
C18:2*n*-6	49.06 ± 2.32^{ac}	49.56 ± 2.88^{a}	41.93 ± 5.74^{b}	44.83 ± 4.21^{bc}
18:2*cis*-9, *trans*-11 (CLA)	0.15 ± 0.15^{ac}	0.4 ± 0.12^{b}	0.07 ± 0.03^{a}	0.29 ± 0.03^{bc}
C18:3*n*-3	2.81 ± 0.46^{a}	2.44 ± 0.45^{a}	10.19 ± 2.36^{b}	12.56 ± 2.02^{b}
C18:3*n*-6	0.6 ± 0.22^{a}	0.41 ± 0.09^{ac}	0.3 ± 0.12^{bc}	0.12 ± 0.01^{b}
C18:4*n*-3	0.04 ± 0.02^{ab}	0.04 ± 0.02^{a}	0.07 ± 0.02^{b}	0.03 ± 0.01^{a}
C20:2*n*-6	0.18 ± 0.13^{a}	0.36 ± 0.1^{b}	0.15 ± 0.08^{a}	0.19 ± 0.04^{a}
C20:3*n*-6	1.96 ± 0.53^{a}	1.7 ± 0.5^{a}	1.19 ± 0.28^{b}	0.7 ± 0.11^{c}
C20:4*n*-6	2.25 ± 1.2^{ab}	2.7 ± 0.85^{b}	1.68 ± 0.21^{c}	1.9 ± 0.29^{ac}
C20:5*n*-3	0.53 ± 0.13^{a}	0.56 ± 0.14^{a}	1.43 ± 0.29^{b}	1.44 ± 0.29^{b}
C22:4*n*-6	0.37 ± 0.18^{a}	0.42 ± 0.15^{a}	0.14 ± 0.05^{b}	0.15 ± 0.07^{b}
C22:5*n*-3	1.22 ± 0.23^{a}	1.19 ± 0.39^{a}	1.67 ± 0.47^{b}	1.98 ± 0.48^{b}
C22:6*n*-3	0.06 ± 0.02^{a}	0.12 ± 0.02^{ac}	0.12 ± 0.03^{bc}	0.23 ± 0.09^{b}
Sum PUFA	59.86 ± 1.95	59.9 ± 3.15	59.08 ± 6.6	64.43 ± 4.83
Sum *n*-3 PUFA	5.29 ± 1.33^{a}	4.63 ± 1.32^{a}	13.58 ± 2.73^{b}	16.43 ± 2.13^{b}
Sum *n*-6 PUFA	54.49 ± 2.57^{a}	55.2 ± 2.74^{a}	45.45 ± 5.81^{b}	47.95 ± 4.16^{b}

Values are % of total fatty acids, expressed as means ± SD. The one way ANOVA (Holm-Sidak method) or ANOVA on ranks (Dunn's Method) for pairwise multiple comparisons were used for comparing means. Values with different superscripts on the same row are significantly different ($P < 0.05$). Diet groups are CTRL: coconut oil; EFA: linseed oil + safflower oil; CLA: Lutalin® and EFA + CLA: linseed oil + safflower oil + Lutalin®.

concentrations as compared to the control (**Figure 2A**). *Cis-9, trans-11* CLA also reduced E2 significantly ($P < 0.05$) at 30 and 60 μM as compared to the control (**Figure 2B**). P4 concentration was significantly reduced in GC treated with ALA at 20 and 40 μM (**Figure 2A**) whereas, P4 was only reduced ($P < 0.05$) by *cis-9*, *trans-11* CLA at 60 μM (**Figure 2B**).

Cell Morphology and Gene Expression

Microscopic images taken on day 8 of *in vitro* culture showed that ALA and *cis-9*, *trans-11* CLA treatment induced intracellular lipid droplet accumulation in GC in particular at higher concentrations (**Figures 3A,B**). Further, RT-qPCR data revealed that transcript abundance of the fatty acid translocase *(CD36)*

TABLE 2 | Effects of dietary essential fatty acids on bovine *in vitro* embryo development.

Diet group (No. of cows)	No. of oocytes (*n*)	(%) Cleavage rate (*n*)	(%) Blastocyst rate (*n*)	Total cell number (*n*)
CTRL (7)	61	34 ± 8.1 (20)	23.5 ± 11.7 (6)	71.2 ± 12.1 (5)
EFA (7)	64	51.7 ± 9.3 (33)	17.4 ± 7.9 (7)	92.1 ± 10.1 (6)
CLA (8)	65	53.3 ± 5.1 (35)	26.1 ± 11.2 (12)	124.9 ± 18.9 (12)
EFA + CLA (8)	70	36.2 ± 10.6 (26)	15.2 ± 8.4 (7)	121 ± 13.2 (7)

Values are means ± SEM. CTRL: coconut oil; EFA: linseed oil + safflower oil; CLA: Lutalin®; EFA+CLA: linseed oil + safflower oil + Lutalin®.

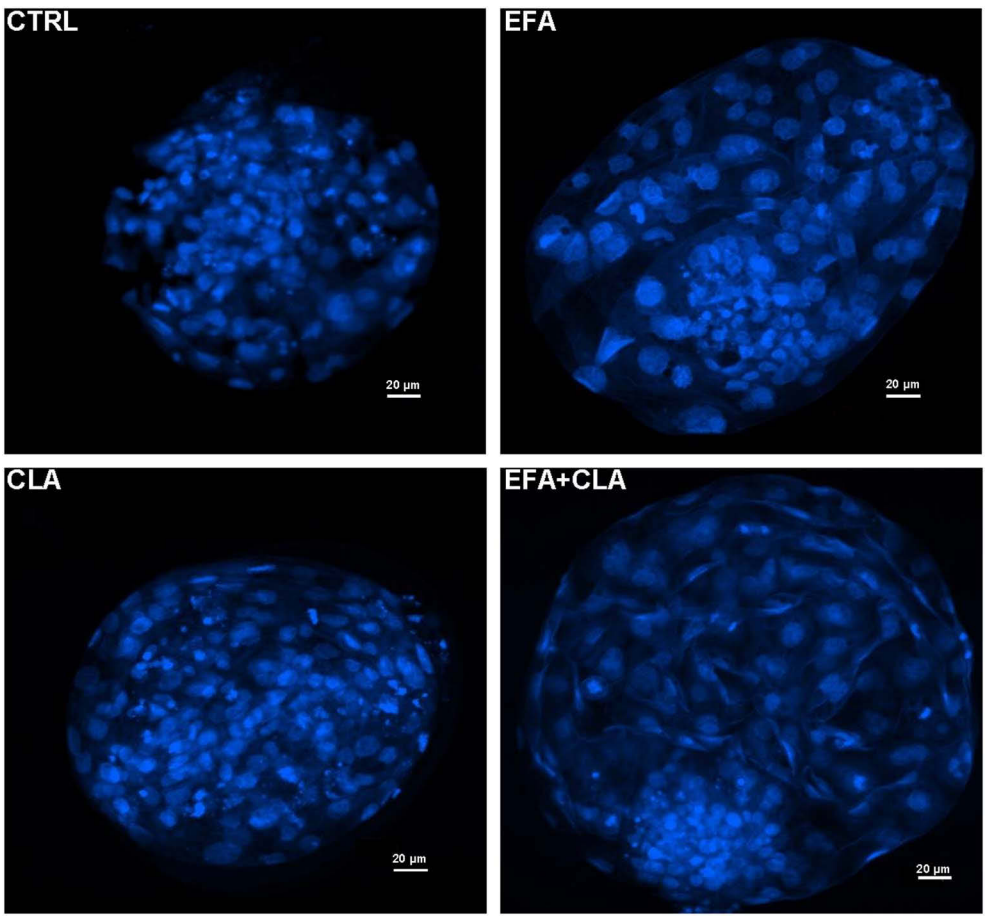

FIGURE 1 | Fluorescence images of Hoechst 33258 stained blastocyst nuclei generated from different dietary groups. CTRL: coconut oil; EFA: linseed oil + safflower oil; CLA: Lutalin®; EFA + CLA: linseed oil + safflower oil + Lutalin®.

was strongly up-regulated by both ALA at 40, 80 μM (**Figure 3C**) and *cis-9*, *trans-11* CLA at 60μM (**Figure 3D**). ALA in GC significantly downregulated ($P < 0.05$) the key transcripts of estradiol production, steroidogenic acute regulatory protein (*STAR*) at all concentrations, cytochrome P450 Family 19 subfamily A member 1 (*CYP19A1*) at (40 and 80 μM), the follicle stimulating hormone receptor (*FSHR*) at 20 and 80 μM and the luteinizing hormone/choriogonadotropin receptor (*LHCGR*) at 80 μM (**Figures 4A,B**). While GC treated with cis-9, trans-11 CLA significantly downregulated ($P < 0.05$) *STAR* at 60 μM, *CYP19A1* at 30 and 60 μM, and both *FSHR* and *LHCGR* at 60 μM (**Figures 5A,B**). Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 *(HSD3B1)* gene expression remained unaltered in ALA (**Figure 4A**) treated GC while it was

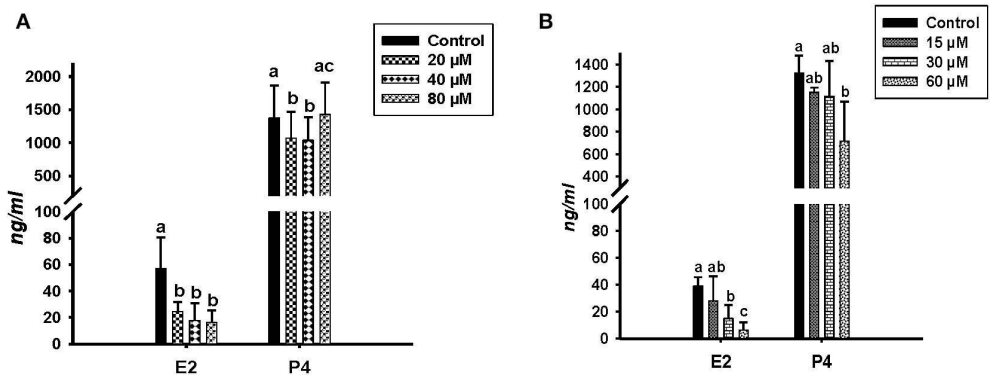

FIGURE 2 | Effects of **(A)** ALA (20, 40, 80 μM) ($n = 5$) and **(B)** *cis-9*, *trans-11* CLA (15, 30, 60 μM) ($n = 3$) on 17β-Estradiol (E2) and progesterone (P4) concentrations (ng/ml) in conditioned media of cultured GC. Data are shown as means ± SEM; different lower case letters indicate significant differences between treatments ($P < 0.05$).

upregulated significantly in *cis-9*, *trans-11* CLA (60 μM) treated GC (**Figure 5A**). The mRNA expression of cyclin D2 (*CCND2)* was significantly downregulated by ALA in GC at 20 and 80 μM (**Figure 4C**), while *cis-9*, *trans-11* CLA downregulated *CCND2* at 60 μM ($P < 0.05$) as compared to the control (**Figure 5C**). Proliferating cell nuclear antigen (*PCNA)* mRNA expression remained unaltered at all tested concentrations in both ALA and *cis-9*, *trans-11* CLA treated GC (**Figures 4C**, **5C**). The GC identity marker forkhead Box L2 (*FOXL2)* was significantly downregulated by ALA at 20 and 80 μM (**Figure 4D**) while *cis-9*, *trans-11* CLA downregulated the same at all concentrations as compare to control (**Figure 5D**). In contrast, SRY-Box 9 (*SOX9*) gene expression was upregulated significantly (**Figure 4D**) in ALA treated GC but seemed slightly affected in *cis-9*, *trans-11 CLA* treated GC (**Figure 5D**).

Cell Viability

Flow cytometry analysis of Annexin V-FITC/PI stained cells showed no significant change in percentage of viable, apoptotic or dead cells in GC treated with different concentrations of ALA as compared to the control. Whereas, GC treated with *cis-9*, *trans-11* CLA at 60 μM resulted in higher percentage of apoptotic cells (16.3 ± 3.1%) compared to control (10.5 ± 3%). Dot plots of Annexin V and propidium iodide (PI) staining generated during a typical flow cytometry experiment as shown in **Figure 6**. The percentage of dead cells in GC treated with *cis-9*, *trans-11* CLA at 30 and 60 μM (12.2 ± 2.2% and 12.4 ± 1.5%, respectively) were significantly higher as compared to the control (8 ± 1.6%). These results were well reflected by the percentage of viable cells going significantly down to 67.5 ± 2.8% in GC treated with 60 μM *cis-9*, *trans-11* CLA as compare to 78.3 ± 1.8% in the control (**Table 3**).

DISCUSSION

Dietary lipid supplementation is supposed to improve fertility in dairy cows by increasing the size of the ovulatory follicle, plasma concentration of P4 and lifespan of CL (25) though, the influence on reproductive performance is by far not fully understood. In the present study, we analyzed the effects of dietary essential fatty acids on oocyte competence and GC function in bovine.

Dietary Supplementation and Embryo Development

Following dietary supplementations, as expected we observed a higher percentage of ALA in the FF of EFA and EFA + CLA diet fed cows, while the percentage of the *cis-9*, *trans-11* CLA was higher in the FF of CLA and EFA+CLA diet fed cows as compared to the CTRL diet fed cows. It has been reported that cows when fed with encapsulated fats containing flaxseed oil had 5-fold higher concentrations of ALA in the FF and GC with increased numbers of follicles as compare to cows fed with encapsulated fats containing sunflower oil and control diet. Also, the cleavage rates in cows fed with flax seed oil were higher than in cows fed with a control diet (26). Similarly, cows fed with flaxseed oil contained higher amounts of ALA in FF, GC, and COCs and the percentage of oocytes that developed to blastocysts was also higher in flaxseed and fish oil fed cows as compared to cows that were fed saturated fats (27). In the present study we found higher proportions of ALA and *cis-9*, *trans-11* CLA in FF of EFA, CLA, and EFA + CLA diet group cows, however, these did not lead to significant improvements in the cleavage and blastocyst rates or TCN count. Our results revealed that neither EFA, CLA, or EFA + CLA diet supplements could improve the cleavage and blastocyst rates of *in vitro* generated embryos as compared to the CTRL diet supplement.

In previous studies, Holstein cows when fed with diets enriched in PUFA especially 18:2 and 18:3 FA despite improving dominant follicle size and CL volume failed to improve both cleavage and blastocyst rates of *in vitro* produced embryos (15). Similarly, feeding soybean oil (high in LA), or linseed oil (high in ALA) to lactating cows could not improve the blastocyst rate or TCN count of *in vitro* produced embryos compared to rumen inert fat (RIF) diet (28). Recent studies have provided new insights in effects of specific *in vitro* FA supplementation on oocyte maturation, cleavage and blastocyst rates. Supplementing *cis*-9, *trans*-11 or *trans*-10, *cis*-12 CLA

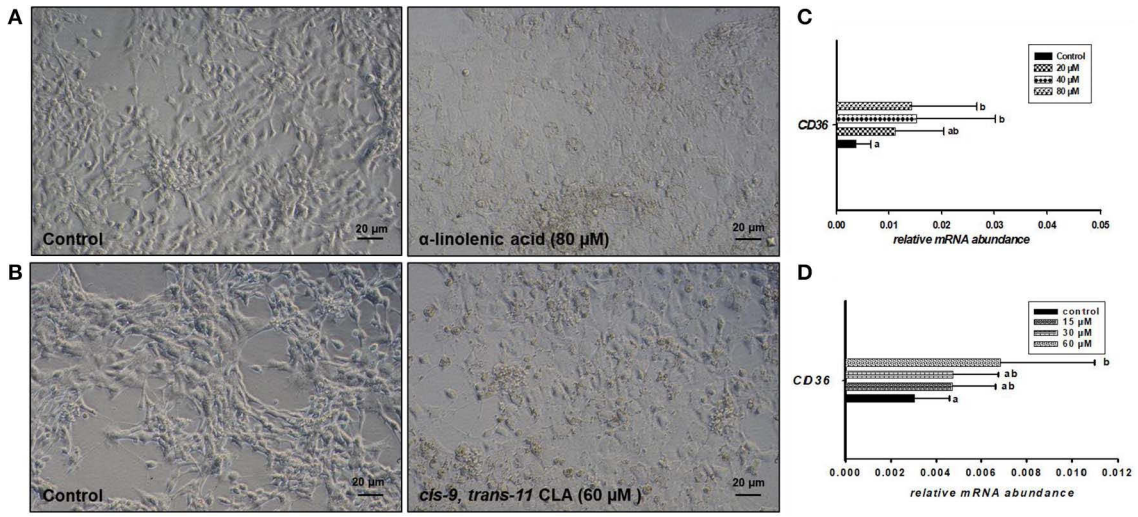

FIGURE 3 | Effects of **(A)** ALA (80 µM) and **(B)** *cis-9*, *trans-11* CLA (60 µM) on the morphology of cultured GC. Photomicrographs were taken with a Nikon TMS-F inverted microscope. Effects of **(C)** ALA (20, 40, 80 µM) ($n = 5$) and **(D)** *cis-9*, *trans-11*CLA (15, 30, 60 µM) ($n = 3$) on *CD36* mRNA abundance. Gene expression was normalized to *RPLPO* transcripts. Data are shown as means ± SEM; different lower case letters indicate significant differences between treatments ($P < 0.05$).

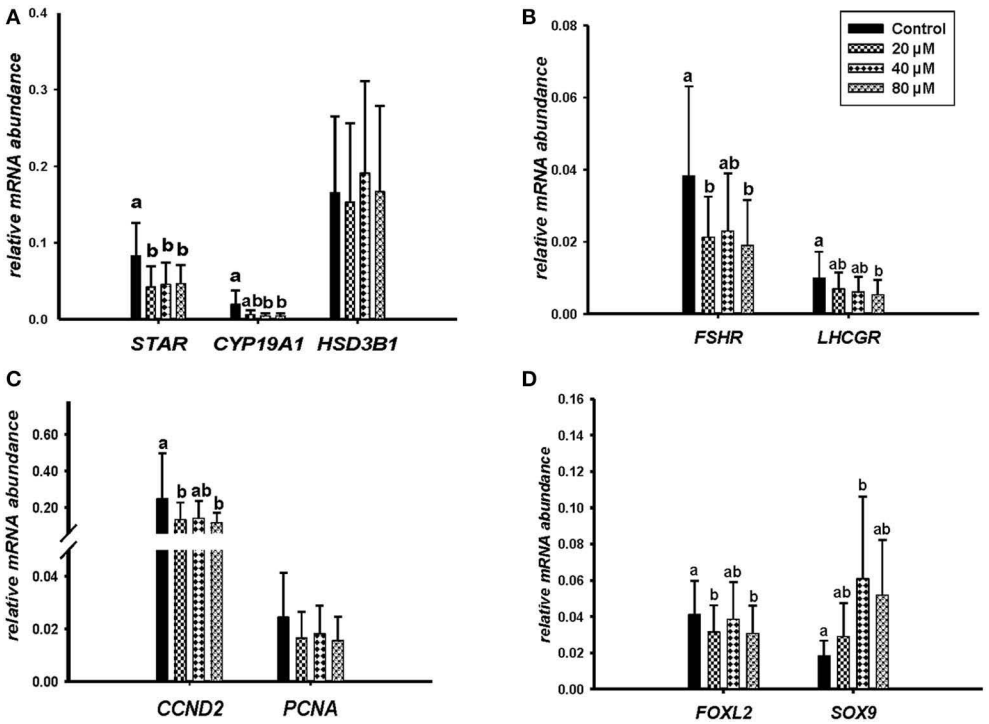

FIGURE 4 | Effects of ALA (20, 40, 80 µM) on the expression of **(A)** key genes of steroidogenesis *STAR, CYP19A1, HSD3B1,* **(B)** gonadotrophin receptors *FSHR, LHCGR*, **(C)** cell proliferation *CCND2, PCNA*, and **(D)** on GC and sertoli cell markers *FOXL2, SOX9.* Gene expression was normalized to *RPLPO* transcripts. Data are shown as means ± SEM; different lower case letters indicate significant differences between treatments ($n = 5$, $P < 0.05$).

isomer could not improve IVP, but embryos generated under *cis*-9, *trans*-11 CLA (100 µM) before vitrification were of improved quality (29). *In vitro* supplementation of ALA (25 µM) during IVM increased the porcine oocyte cleavage rate but at higher concentration of ALA (50 µM), though enhancing the nuclear maturation of oocytes it could not improve the cleavage, blastocysts rate or TCN. This suggests that high concentrations of ALA can adversely affect *in vitro* embryo development (30). In the present study we speculate that adverse effects of EFA and CLA diets on oocyte competence could be associated with

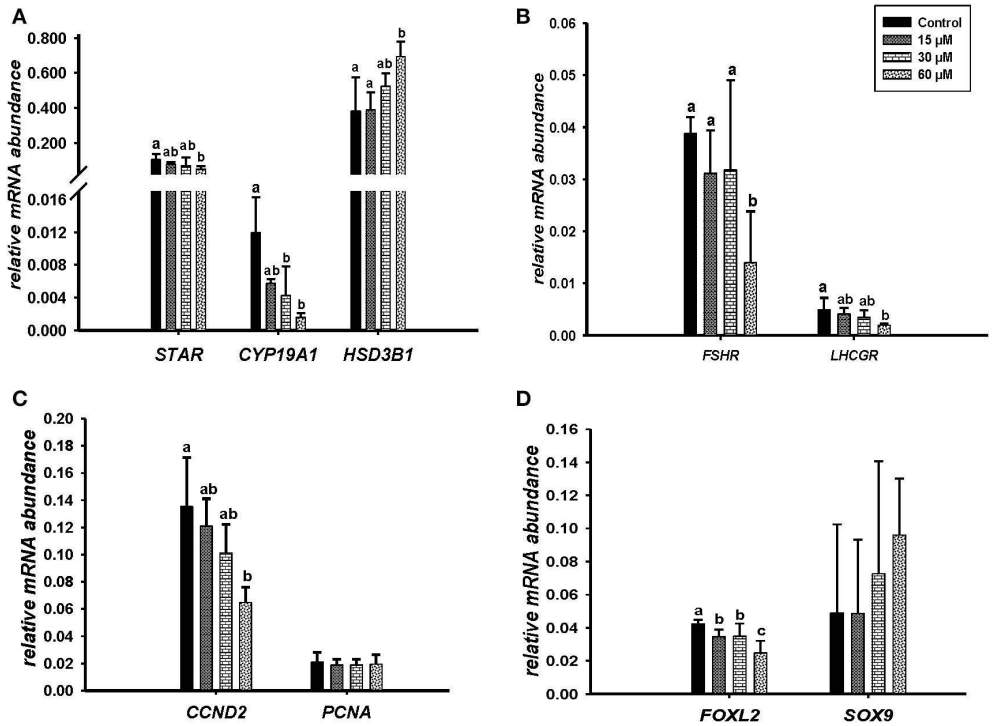

FIGURE 5 | Effects of *cis-9*, *trans-11* CLA (15, 30, 60 µM) on the expression of **(A)** key genes of steroidogenesis *STAR, CYP19A1, HSD3B1,* **(B)** gonadotrophin receptors *FSHR, LHCGR*, **(C)** cell proliferation *CCND2, PCNA*, and **(D)** on GC and sertoli cell markers *FOXL2, SOX 9.* Gene expression was normalized to *RPLPO* transcripts. Data are shown as means ± SEM; different lower case letters indicate significant differences between treatments ($n = 3$, $P < 0.05$).

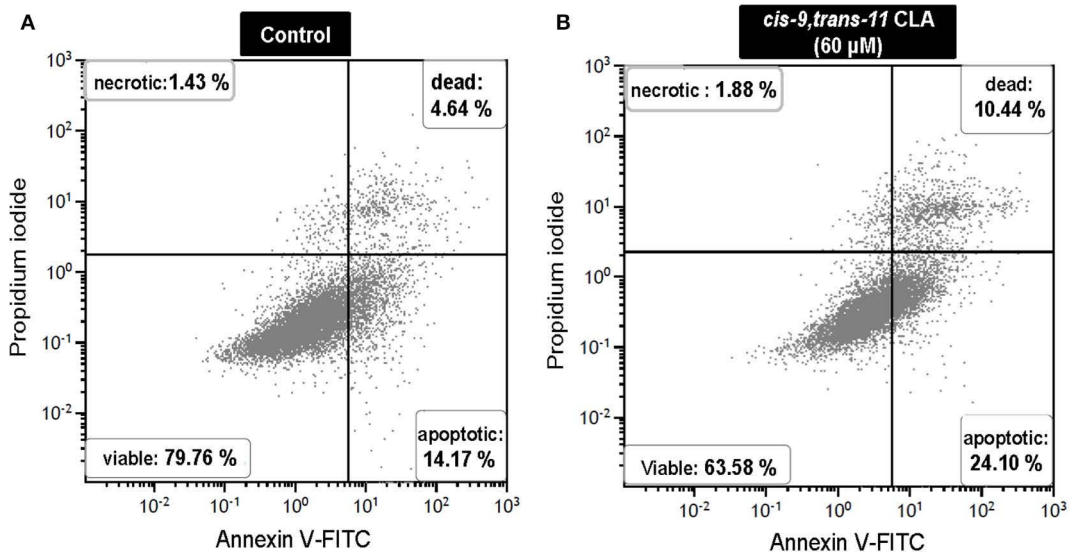

FIGURE 6 | Representative dot plots of Annexin V and propidium iodide (PI) staining generated during a flow cytometry experiment. Annexin V and PI staining in **(A)** control and **(B)** *cis-9*, *trans-11* CLA (60 µM) cultured GC. Percentage of cells stained in each quadrant is listed in the corner of each quadrant.

higher proportions of ALA and *cis-9*, *trans-11* CLA as observed in the FF. Previous studies have documented the decisive role of FF components in determining oocyte quality and fertility in bovine and humans (31, 32). However, the small sample size could be a limitation in achieving decent blastocyst rates. Another plausible reason could be the experimental approach of oocyte retrieval from subordinate follicles. Since the ovaries were obtained from cows at different oestrous stage, which might affect their oocyte development competence as dominant follicle might suppress the subordinate follicles. And thus, the sliced

TABLE 3 | Cell viability as determined by Annexin V-FITC/PI staining assay in bovine *in vitro* granulosa cells.

	α-linolenic acid (ALA)				*cis-9, trans 11* CLA			
Treatments	**control**	**20 μM**	**40 μM**	**80 μM**	**control**	**15 μM**	**30 μM**	**60 μM**
Viable cells (%)	76.4 ± 0.5	70.4 ± 4.1	66.9 ± 2.6	73.7 ± 1.7	78.3 ± 1.8^{a}	75.6 ± 3.9^{ab}	71.7 ± 3.7^{ab}	67.5 ± 2.8^{b}
Apoptotic cells (%)	10.5 ± 3.5	10.8 ± 1.4	12.9 ± 5.4	10.6 ± 2.9	10.5 ± 3.0^{a}	9.8 ± 2.5^{ab}	11.9 ± 2.2^{ab}	16.3 ± 3.1^{b}
Dead cells (%)	9.4 ± 2.5	14.6 ± 4.4	14.1 ± 1.8	11.7 ± 1.0	8 ± 1.6^{a}	11.2 ± 2.6^{ab}	12.2 ± 2.2^{b}	12.4 ± 1.5^{b}

Data analyzed by one-way repeated measure ANOVA, multiple comparison vs. control (n = 3; Dunnett's method). Values are means ± SEM. Different lower case letters on the same row indicate significant differences between treatments (P < 0.05).

follicles might not reflect the environment of the dominant preovulatory follicle. Furthermore, the selective dietary uptake of EFA and CLA supplementation leading to increased proportions of ALA and *cis*-9, *trans*-11 CLA in the follicular fluid might adversely affect developmental competence of oocytes residing within the follicles.

ALA and *cis-9, trans-11* CLA Alter GC Morphology and the Expression of Identity Marker Genes

In vitro supplementation of ALA and *cis-9, trans-11* CLA induced significant alterations of the morphology of cultured GC. The morphology of the cells was similar to oleid acid (OA) treated GC that we already reported earlier (33). These morphological changes are in line with the observation of an increased mRNA abundance of fatty acid translocase, *CD36,* which is known to mediate fatty acid uptake (34). The observed results are also consistent with reports in other mammalian cell lines. *Cis-9, trans-11* CLA induced lipid accumulation in human macrophages and in mice 15P-1 cell lines (testicular cells) along with an elevated expression of *CD36* (35, 36). ALA enriched diets increased the transport of lipids into resting skeletal muscles in conjunction with increased sarcolemmal ω-3 PUFA content and *CD36* protein expression in rats (37). This suggests that both ALA and *cis-9, trans-11* CLA are well-accumulated in mammalian cells either *in vitro* or *in vivo* as diet supplements.

The transcriptional regulators, *FOXL2* and *SOX9* are well-associated to each other (38) as *FOXL2* prevents transdifferentiation of the adult ovary to testis. Inducible deletion of *FOXL2* in adult ovarian follicles can upregulate the testis-specific marker gene *SOX9* (39). Previously, we reported that OA markedly reduced the transcription of *FOXL2* and increased *SOX9* in cultured GC (40). A similar regulation was observed in the present study with ALA *and cis-9, trans-11 CLA* supplementation decreasing the mRNA abundance of *FOXL2* and upregulating *SOX9* transcripts. Apart from being an GC identity marker *FOXL2* is also known to regulate ovarian steroid metabolism (41). The presented results clearly indicate that both, ALA and *cis*-9, *trans-11* CLA modulate the *FOXL2* transcriptional activity. Thus, the role of *FOXL2* in regulating the lipid metabolism in terms of promoting lipid droplet formation in GC upon ALA *and cis-9, trans-11* CLA treatment could be well-anticipated from the presented data. The results suggest that both ALA and *cis-9, trans-11 CLA* impose alike effects on the transcript levels of *FOXL2* in GC which might further affect other downstream target molecules involved in normal GC function.

ALA and *cis-9, trans-11* CLA Modulate GC Steroidogenesis

For normal GC function, steroidogenesis is initiated by the transportation of free cytoplasmic cholesterol into mitochondria by the protein *STAR* (42). Successive steps are then catalyzed by cholesterol side chain cleavage enzyme encoded by cytochrome P450 side-chain cleavage enzyme (*CYP11A1)*, by 3 beta hydroxyl steroid dehydrogenase transcribed from *HSD3B1*, and by *CYP19A1* encoding the key enzyme of E2 synthesis (43). Steroidogenesis is a vital part of folliculogenesis as studied in knockout mouse models (44). Also FSHR and LHCGR play a vital role during follicle maturation (45).

In the present study, we determined the effects of ALA and *cis-9, trans-11* CLA on steroid hormone production, on transcriptional activity of key genes involved in steroidogenesis (*CYP19A1, STAR,* and *HSD3B1)* and gonadotropin hormone signaling (*FSHR* and *LHCGR)* in cultured GC. The transcript level of *STAR* as well as the E2 production was significantly downregulated by both ALA and *cis-9, trans-11* CLA. Most likely, the reduction in E2 concentrations can be attributed to reduced transcription of *CYP19A1* as observed after ALA and *cis-9, trans-11* CLA treatment. CLA has been shown to be very potent in reducing *CYP19A1* expression and E2 production in buffalo GC as well (46). Another PUFA, arachidonic acid (AA) at 50 μM stimulates the proliferation of bovine GC by activating both extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt signaling pathway, however, higher dose decreases E2 secretion and downregulates the mRNA abundance of *CYP19A1*, *FSHR*, *HSD3B1,* and *STAR* in cultured bovine GC (47). This, suggest that PUFA at higher levels might modulate steroidogenesis by intracellular signaling pathways that regulate target gene expression. FSH is known to induce the expression of *LHCGR* via protein kinase A (PKA) and phosphoinositide3-kinase (PI3K) pathways in rat GC (48). *LHCGR* is highly expressed in GC during the pre-ovulatory stage to enable responsiveness to the LH surge thus leading to ovulation, oocyte maturation and CL formation (49). The down-regulation of *FSHR* and *LHCGR* by both FA at higher concentrations might affect follicle stimulating hormone (FSH) signaling and steroidogenesis in GC as suggested by our results. *CYP11A1* converts cholesterol into pregnenolone and *HSD3B1* converts pregnenolone into P4 (50). *HSD3B1* gene expression though remained unaltered in GC treated with ALA but P4 production was reduced. Similar effects on P4 production

have been observed in primary goat GC treated with ALA at 100 μM (51). Surprisingly, *cis-9, trans-11* CLA significantly upregulated *HSD3B1* gene expression while reducing the P4 concentration in conditioned media. This outcome might be due to negative feedback effects of P4 production on *HSD3B1* transcription. Together, these results suggest that high levels of ALA or *cis-9, trans-11* CLA in FF can adversely affect GC by reducing both E2 and P4 production, which in turn could result in compromised ovarian cyclicity and impaired fertility in lactating cows. This is also in line with our recent study, where we could show that increased concentrations of OA in the follicular fluid can actually impair the functionality of GC *in situ*, thus obviously suppressing ovulation (21).

cis-9, trans-11 CLA Induces Apoptosis in GC

It is well-documented that higher E2 levels are capable of protecting GC from Fas ligand induced apoptosis and promote the cell transit from G1 to S phase with increased *CCND2* expression (52, 53). In our results, GC morphology was altered upon ALA and *cis-9, trans-11* CLA treatment, and both ALA and *cis-9, trans-11*CLA could down-regulate *CCND2* mRNA expression. However, only *cis-9, trans-11*CLA could elicit significant apoptotic effects in GC. As reported earlier, CLA is known to induce apoptosis in human, mouse and rat cell lines (54–57). *CCND2* mRNA expression is FSH dependent as reported in FSHR null mutant mice exhibiting decreased *CCND2* mRNA levels (58). This goes well in line with our observation that *cis-9, trans-11* CLA treated cells not only reduced E2 production, but also reduced *FSHR* mRNA expression followed by a steep downregulation of *CCND2* mRNA expression.

In contrast, ALA despite of reducing the E2 levels and *CCND2* mRNA abundance did not induce any apoptotic effects in GC. This observation suggests the importance of FSH signaling during cell cycle regulation of GC. However, proliferating cell nuclear antigen (PCNA) gene expression remained unaltered in GC treated with both ALA and *cis-9, trans-11* CLA. According to previous immunocytochemistry studies different *cis-9,trans-11* CLA concentrations at different time periods could significantly decrease the *PCNA* expression and inhibit cell growth and proliferation in mammary cancer cells (MCF-7 cells) (59) and in gastric adenocarcinoma cell lines (SGC-7901) (60). However, these effects were only observed on translation/protein levels as changes of transcript abundance were not determined.

CONCLUSION

We investigated the effects of dietary essential fatty acids on bovine oocyte competence and GC functionality. Essential fatty acid supplementation could not improve the embryo IVP in spite of a substantial increase of ALA and *cis-9, trans-11* CLA in the follicular fluid. Further, the study clearly suggests that GC morphology and functionality could be considerably affected with increased follicular proportions of ALA and *cis9, trans-11*CLA particularly constraining steroidogenesis in GC. *In vivo* this may result in a compromised ovarian cyclicity and impaired fertility.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by federal state of Mecklenburg Western-Pommerania, Germany LALLF M-V TSD 7221.3-1-038/15.

AUTHOR CONTRIBUTIONS

HH designed the dietary supplements for the cows. AV and VR performed the ovarian follicular fluid collection. DD and TV executed the fatty acid analysis. JS and AS executed the IVF experiments. AS, VB, and JV designed and executed the *in vitro* cell culture experiments. AS and JV primarily wrote the manuscript, with all co-authors making significant contribution to the final manuscript.

FUNDING

This study was financially supported by FITCOW (Geschäftszeichen: 313-06.01—28-1—79.003-15) project and funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme.

ACKNOWLEDGMENTS

All authors appreciate Swanhild Rodewald, Bianka Drawert, Veronika Schreiter, Maren Anders, Christian Plinski, Bergit Jentz, and Maria Dahm for their technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00087/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Study III.

Non-Esterified Fatty Acids in the Ovary: Friends or Foes?

Baddela V.S, Sharma A, Vanselow J. *Reproductive Biology and Endocrinology* (2020). (**Accepted on 25.05.2020 for publication**)

Brief summary:

The review highlights common metabolic diseases that arise due to excessive lipolysis, thus leading to elevated levels of NEFAs in the body fluids of humans and livestock species. Increased NEFA levels in the FF markedly affect the function of ovarian elements especially of GCs, cumulus cells and oocytes. However, fatty acids differing in their chemical properties such as being saturated or unsaturated might carry differential effects on ovarian elements as suggested by various, partially contradictory studies. SFAs are known to deteriorate oocyte maturation and developmental competence by initiating the apoptotic cascade in granulosa and cumulus cells of ovarian follicles in different species. While, UFAs at elevated level has been found to modulate steroidogenesis and proliferation in follicular cells. Remarkably, OA has been widely reported to cause PCOS in both human and bovine. In general, the review demonstrates the modulatory effects of NEFAs on ovarian function and their possible impact on reproductive efficiency in human and livestock species.

Non-Esterified Fatty Acids in the Ovary: Friends or Foes?

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Abstract:

A majority of common metabolic diseases can result in excessive lipolysis, leading to elevated levels of non-esterified fatty acids (NEFAs) in the body fluids. In females, increased NEFA levels in the follicular fluid markedly alter the functions of intrafollicular cells such as granulosa cells (GCs) and oocytes. Therefore, elevated levels of NEFAs have been suggested to be a significant player of subfertility in females of both human and economically important animal species such as cattle, buffalo, sheep, pig, chicken, and dog. However, the effects imposed by saturated and unsaturated fatty acids (SFAs and UFAs) on ovarian follicles are controversial. The present review emphasizes that SFAs induce apoptosis in granulosa and cumulus cells of ovarian follicles in different species. They further could adversely affect oocyte maturation and developmental competence. Many types of UFAs affect steroidogenesis and proliferation processes and could be detrimental for follicular cells, especially when at elevated concentrations. Interestingly, monounsaturated fatty acids (MUFAs) appear to contribute to the etiology of the polycystic ovarian syndrome (PCOS) as they were found to induce the transcription and translation of the androgenic transcription factor SOX9 while downregulating its estrogenic counterpart FOXL2 in GCs. Overall, this review presents our revised understanding of the effects of different fatty acids on the female reproductive success, which may allow other researchers and clinicians to investigate the mechanisms for treating metabolic stress-induced female infertility.

Keywords: NEFAs, granulosa cells, oocyte, ovary, metabolic diseases

1. Introduction to the follicular physiology:

Ovarian follicles are a large but limited pool of highly complex miniature structures in the ovary. They are originally assembled in the form of primordial follicles containing a single layer of flattened pre-granulosa cells surrounding a dormant immature oocyte. Each primordial follicle is enclosed by a basement membrane, which separates the follicle from the rest of the ovarian stroma throughout follicular development. Some of the primordial follicles commence growth as soon as they are formed and develop into primary and some eventually into secondary follicles containing distinct cuboidal shaped granulosa cells (GCs) with a larger oocyte in the center. However, a majority of primordial follicles stay in a quiescent stage for months or years before commencing further development [1]. Follicles with a single layer of GCs are called primary follicles, and those with multiple GCs layers are called secondary follicles. The development of primordial follicles into secondary follicles takes place in a gonadotropin independent manner. However, expression of the gonadotropin receptor, i.e., follicle-stimulating hormone receptor (FSHR) can already be detected in the GC layer of primary follicles [2]. Therefore, the development from primary to secondary follicles is argued to be a gonadotropin sensitive mechanism. In secondary follicles, additional layers such as the theca interna and theca externa start appearing on the outer side of the basement membrane and contribute to follicular development [3]. The developmental events from secondary follicles into preovulatory follicles take place in a gonadotropin dependent manner as the GCs of ovarian follicles can be stimulated by follicle stimulating hormone (FSH). Together with hepatic insulin-like growth factor 1 (IGF-1), FSH induces proliferation and steroidogenesis in GCs and supports the formation of the follicular

antrum [4]. Antral ovarian follicles are also called tertiary follicles and their fate is largely determined by the function of GCs. A pool of small antral follicles are recruited into the ovarian cycle in every menstrual or estrous cycle. In response to FSH and IGF-1, one of the recruited antral follicles becomes the dominant follicle and produces ample amounts of the female sex hormone 17β-estradiol (E2), which in turn induces the release from the anterior pituitary of the second gonadotropin hormone i.e. luteinizing hormone (LH). The antral follicle that has achieved optimal growth and is present at the precise time window during the follicular phase of the ovarian cycle survives as a dominant follicle in each ovarian cycle. The pre-ovulatory LH surge triggers the release of the mature oocyte from the fully developed dominant follicle for fertilization. The somatic cells of the theca and granulosa layers from the ovulated follicle subsequently differentiate into the corpus luteum (CL) (Figure 1). In mono-ovulatory animals such as humans and bovines, only one of the recruited follicles will be selected to become a dominant follicle while others undergo atresia. In contrast, in poly-ovulatory animals such as pigs, dogs, and rodents, multiple dominant follicles are selected to undergo ovulation per ovarian cycle.

In addition to timely endocrine stimulations, follicular development depends on the health and energy status of the animal. It has been shown that folliculogenesis is dramatically affected during periods of limited food availability (e.g., malnutrition or fasting) or increased energy demands (e.g., during lactation and negative energy balance (NEB)), which are not met by compensatory food intake [5-7]. Interestingly, over-nutrition associated metabolic diseases, such as obesity and diabetes 2, could also impair the development of ovarian follicles in many different species, including humans, mice, and rats [8, 9]. At least one biochemical factor that has been commonly

identified as a characteristic marker for both under- and over-nutrition metabolic conditions is the elevated levels of non-esterified fatty acids (NEFAs) in the circulation. Significantly higher serum concentrations of NEFAs were found in heifers after short term fasting [7] and in dairy cows during negative energy balance [10, 11], as well as in obese women [12]. Furthermore, some reports show that polycystic ovarian syndrome (PCOS), which is argued to be a metabolic disease, is also characterized by the presence of excess amounts of NEFA in body fluids [13-15]. However, a significant increase of NEFA concentrations was not consistently found [13, 16], indicating that these diseases are not always associated with elevated NEFA levels. This might be possibly due to differential genetic and environmental factors (Tables1 and 2) and needs future investigation.

2. Fatty acids

Fatty acids (FAs) are carboxylic acids with an aliphatic chain of different lengths and saturation levels. FAs are broadly classified into two categories: 1) saturated fatty acids (SFAs) and 2) unsaturated fatty acids (UFAs). SFAs contain only single bonds between the carbons of their aliphatic chain, e.g. palmitic acid (PA, 16:0) and stearic acid (SA 18:0), whereas UFAs contain one or more double bonds. e.g., oleic acid (OA 18:1) and linoleic acid (LA 18:2) [17]. Furthermore, UFAs can be classified into 2 subcategories: i) monounsaturated fatty acids (MUFAs), which contain only one double bond, e.g., palmitoleic acid (16:1) and OA (18:1), and ii) polyunsaturated fatty acids (PUFAs), containing two or more double bonds in the aliphatic chain. UFAs are also classified on the basis of the position of the first double bond starting from the methyl end of the carbon chain. Omega-3 FAs have the first double bond at the third carbon atom and

include alpha-linolenic acid (ALA 18:3 n–3), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Omega-6 FAs have the first double bond at the sixth carbon atom, which produces LA and its derivative arachidonic acid (AA). Omega-9 FAs have the first double bond at the ninth carbon atom (e.g., OA). The ω-6 and ω-3 FAs such as LA and ALA are called essential fatty acids (EFA) as humans cannot synthesize them *de novo*. LA is the parent FA for the remaining ω-6 EFAs, whereas ALA is the parent FA for the remaining ω-3 EFAs. Therefore, humans need dietary supplementation with LA and ALA to produce higher-order UFAs such as arachidonic acid (C20:4) and docosatetraenoic acid (C22:4).

FAs are essential constituents of all living cells and have significant roles as components of biomembranes, cell signaling (steroid hormones and prostaglandins), and energy substrates (e.g., in the form of di- or tri- acylglycerols). They are widely favored as the preferred form of stored energy because of their low hydrodynamic diameter and the incredibly high amount of energy released upon their oxidation as compared to carbohydrates. During periods of starvation/fasting, de-esterification of FAs from stored lipids of the adipose tissue takes place by the action of a hormone-sensitive lipase, resulting in the temporary elevation of NEFAs in the circulation for coping with the body's energy demands [7, 18]. However, such lipolysis is repressed in healthy animals by the action of insulin, whose levels are increased after an energy-rich meal.

Severe and unregulated lipolysis is a hallmark of various metabolic diseases such as obesity, diabetes 2, NEB, and subclinical ketosis and it causes continuously elevated levels of NEFAs in the body fluids of humans and animals (Table 1 and Table 2) [7, 11, 19]. Elevated levels of NEFAs in the circulation, in turn, enter the follicular fluid and alter

the concentrations in developing ovarian follicles [20]. Valckx et al. 2014 [21] showed that *in vitro* exposure of murine ovarian follicles to elevated levels of NEFAs resulted in the impairment of ovarian steroidogenesis and oocyte competence for fertilization. To further understand the physiological responses under *in situ* conditions, Sharma et al. 2019 [22] took advantage of the ultrasound-guided injection approach to inject NEFAs (oleic acid; C18:1) into the dominant follicles of heifers *in situ*. Interestingly, the NEFA injected animals showed reduced ovulation rates and reduced production of E2 hormone compared to that of the control group of animals (Figure 1). Therefore, it can be implied that the ovarian dysfunction in animals/humans observed during metabolic diseases/conditions can be due to elevated levels of accumulating follicular NEFAs. In the follicular fluid, PA, SA, and OA together contribute up to 45% of the total NEFA concentration. These fatty acid levels can further increase or even double in the follicular fluid during specific metabolic conditions in humans and animals [7, 12, 19]. Therefore, a major emphasis will be given to the effects of these FAs in the following sections.

3. Effects of NEFAs on GC function:

GCs are steroidogenic somatic cells of the ovarian follicle. They play an indispensable role in the nourishment of the oocyte and regulation of the ovarian cycle. Under healthy conditions, GCs of dominant follicles synthesize and secrete ample amounts of E2 upon stimulation with FSH and IGF-1 (Figure 2). The increased systemic levels of E2 trigger the release of LH from the anterior pituitary in a positive feedback mechanism *via* gonadotropin-releasing hormone (GnRH) and induce ovulation. Therefore, compounds that affect the function of GCs could potentially negatively impact female fertility.

FAs have been reported to alter GC function by affecting steroidogenesis, proliferation, and apoptotic processes necessary for follicular development [22-24]. Elis et al. 2015 [25] have shown the mechanisms by which the FA metabolism is linked to GC function in bovines using chemical inhibitors such as etomoxir and C75 (4-methylene-2-octyl-5-oxotetra- hydrofuran3-carboxylic acid). Etomoxir prevents FA oxidation by irreversibly inhibiting the carnitine palmitoyltransferase-1 enzyme. C75 is an inhibitor of the fatty acid synthase complex, thus preventing fatty acid biosynthesis. Both inhibitors decreased the IGF-1 induced proliferation of GCs and affected the phosphorylation of key enzymes of the cellular metabolism such as AMPK (5′ adenosine monophosphate-activated protein kinase) and acetyl CoA carboxylase. These data suggest that basal the FA metabolism (anabolism and catabolism) is essential for GC function and optimal follicular growth.

A growing number of studies describe that an excess amount of NEFAs disrupts the function of GCs during metabolic diseases. One of the apparent effects of elevated NEFA levels is the induction of dramatic morphological alterations in GCs. In addition to the significant impact on the expression of critical genes and hormone production, Yenuganti et al. 2016 [26] and Sharma et al. 2019 [22] reported the formation of foam cell-like structures in cultured bovine GCs upon treatment with increased levels of NEFAs such as OA, PA, and SA; the formation was possibly the result of excessive lipid droplet accumulation. The altered morphology of GCs has been attributed to the active uptake of FAs by GCs *via* the CD36 translocase system. CD36 is an 88-kDa transmembrane glycoprotein with lipid-based ligand binding for low-density lipoproteins (LDL), high-density lipoproteins (HDL), very low-density lipoproteins (VLDL), fibrillar β-

amyloid, and collagen molecules. Expression of CD36 induces the accumulation of conjugated linoleic acids (CLA) in human macrophages and 15P-1 cell lines (testicular cells) of mice [27, 28]. Similarly, CD36 protein expression is increased upon feeding ALA enriched diets to rats promoting the transport of lipids into their resting skeletal muscles [29]. This information suggests that CD36 is actively involved in the uptake of FAs in mammalian cells. Upon cellular uptake, different FAs appeared to elicit different responses, depending on the degree of saturation/unsaturation of FAs.

3.1. Saturated fatty acids:

Accumulating evidence indicates that SFAs have adverse effects on GC function. PA and SA have been found to induce apoptosis in cultured human primary GCs and in human KGN cells in a dose-dependent manner [30]. Particularly, at a concentration of 300 μM, PA was found to cause a significant decline in the expression of the anti-apoptotic protein BCL2 (B-cell lymphoma 2) and an increase in the expression of BAX (BCL2 associated X protein), a pro-apoptotic protein, in human primary GCs. Distinct DNA fragmentation in PA (300 μM) treated GCs confirmed the apoptotic effects of SFAs. However, PA had no apoptotic effects at a concentration of 100 μM, whereas SA could still pose a marginal yet significant decrease in cell viability at 100 μM, which became very severe as the concentration of SFAs was increased. This indicates that elevated concentrations (>100 μM) of SFAs are cytotoxic to human GCs. Similar negative effects of SFAs have been reported in mice, cows, and pigs [22, 31]. Shibahara et al 2020 [32] have recently confirmed the proapoptotic effects of SFAs as they observed induced expression of caspase-3 and C/EBP homologous protein (CHOP) and decreasing Akt phosphorylation in porcine GCs. These effects were

observed in association with reduced cell viability and increased ceramide accumulation.

FAs are well-known ligands for peroxisomal proliferator-activated receptors (PPARs) in mammalian cells. In an interesting experiment, Mu et al. 2001 [30] induced PPAR signaling with a synthetic FA analog, fenofibrate. The results revealed that fenofibrate could not induce apoptosis in human GCs, suggesting that FA induced apoptosis may not be the result of direct interactions of FAs with PPAR in GCs. Interestingly, cellular apoptosis seems to be mediated by metabolites of SFAs as shown in chicken and human GCs. PA-induced apoptosis of GCs is accompanied by increased expression of genes coding for various enzymes of the FA and lipid metabolism, including carnitine palmitoyl transferase-1 (CPT1), serine palmitoyltransferase (SPT), acyl CoA oxidase (ACO), and sphingomyelinase (SMASE) [33]. Therefore, the effects of different chemical inhibitors that could inhibit these enzymes were tested to identify the mechanisms involved in FA induced cell death in GCs. Surprisingly, independent supplementation with triacsin C (fatty acyl CoA synthase inhibitor), imipramine (sphingomyalinase inhibitor), fumonisin B1 (ceramide synthesis inhibitor) and pyrrolidine dithiocarbamate (free radicle scavenger) rescued PA-induced cell death in chicken GCs [33]. Similarly, triacsin-C supplementation was found to significantly decrease both PA and SA induced cell death in human GCs [30]. However, treatment with fumonisin B1 and the nitric oxide synthase inhibitor aminoguanidine, could not inhibit PA-induced apoptosis in human GCs. Together, these data suggest that especially acylated forms of FAs, which are precursor forms of FA oxidation, are detrimental for cell health. Further validation of this phenomenon was provided when human GCs were treated

with acyl derivates of different SFAs and UFAs. Similar to the effects of PA and SA, palmitoyl CoA and stearoyl CoA significantly decreased cell viability in a dose-dependent manner [30]. These observations indicate that excessive β-oxidation of SFAs could be a potential inducer of apoptosis and cell death, possibly by generating an excessive amount of ROS in GCs of different species.

However, PA and SA could not elicit early apoptotic effects as shown by an Annexin V assay in bovine GCs which might be due to increased transcription of FSH signaling induced genes such as cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*), follicle stimulating hormone receptor (*FSHR*), luteinizing hormone/choriogonadotropin receptor (*LHCGR)* as well as enhanced synthesis of E2 by PA and SA [6, 22]. It has been shown that higher E2 levels protect the cells from Fas ligand-mediated apoptosis and induce proliferation by increasing the percentage of cells entering the S phase of the cell cycle [34]. Similar upregulation of steroidogenesis has been reported in bovine adrenal cells exposed to SFAs [35]. However, Vanholder et al. 2005 [6] showed in bovine GCs that both PA and SA could significantly increase the number of dead cells under in-vitro conditions. These contradictory observations can be very likely explained by the opposing effects of SFAs and E2 on apoptosis. On the one hand, apoptosis can be promoted by PA and SA treatment, but on the other hand, these detrimental effects of SFAs can be overridden by increased E2 production in E2 active GC. Therefore, the E2 active state of GCs must be acknowledged while discussing the effects of FA.

SFAs were also found to have detrimental effects on bovine cumulus cells, which are distinct from mural GCs and have a relatively lower steroidogenic ability. *In vitro*

exposure of bovine cumulus-oocyte complexes (COCs) to PA and SA induces endoplasmic reticulum (ER) stress, mitochondrial damage and apoptosis in cumulus cells [36, 37]. ER stress in cumulus cells has been determined by measuring the gene expression of *ATF4* (activating transcription factor 4) and *HSPA5* (heat shock protein family A (Hsp70) member 5) [38]. The expression of *ATF4* and *HSPA5* genes was also up-regulated in mouse COCs exposed to the lipid-rich follicular fluid compared to lipid-deprived follicular fluid [24].

IGF-1 signaling plays a key role in GC steroidogenesis and proliferation [39]. More recently, it was observed that PA inhibits glucose uptake and induces insulin resistance in KGN cells by inhibiting IGF-1 induced Akt phosphorylation while increasing c-Jun N-terminal kinase (JNK) phosphorylation [40] (Figure 2). Furthermore, the inhibition of JNK phosphorylation reversed the PA-induced downregulation of Akt phosphorylation. These results are in line with earlier observations made by Walsh et al. 2012 [41] that NEB like conditions such as acute dietary restriction, which elevate NEFAs levels, could affect the IGF-1 and gonadotropin signaling associated gene expression in GCs, leading to an anovulatory phenotype in animals.

Overall, it is apparent that the metabolites of SFAs could induce apoptosis in GCs of different species which may be ameliorated by inhibitors of FA metabolism and antioxidant molecules. In any case, the E2 active phenotype of GCs may play a key role in the apoptotic response of GCs to elevated SFAs.

3.2. Unsaturated fatty acids:

Similar to SFAs, UFAs are also known inducers of fatty acid transporters, *CD36,* and solute carrier family 27 member 1 in GCs, thus promoting their active uptake [26].

However, in contrast, UFAs show mostly opposite biological effects on GC function as compared to SFAs. It has been shown that UFAs can counteract the cytotoxic effects of SFAs by stimulating triacyl glyceride formation, which leads to a reduction of SFA concentrations available for oxidative processes, the byproducts of which are primarily cytotoxic. [33, 42]. However, MUFAs such as OA have been shown to cause a significant decline in the proliferation of *in vitro* cultured GCs of both human and bovine origin [6, 20, 30]. On the other hand, Sharma et al 2019 [22] reported upregulation of the proliferation marker cyclin D2 (CCND2) in bovine GCs by SFAs such as PA and SA. These effects clearly suggest that different signaling mechanisms are influenced by SFAs and UFAs. This can be partially explained by data from rat skeletal muscle cells [43]. This study showed that OA could induce Akt phosphorylation and also reverse the PA-induced dephosphorylation of Akt by activating the PI3K (phosphoinositide 3-kinase-3-kinase) pathway in skeletal muscle cells. However, the downstream pathways of Akt such as forkhead box protein O1 (FOXO1), glycogen synthase kinase 3 (GSK3) and mammalian target of rapamycin (mTOR), which could hold the key to explaining these observed effects, have not yet been studied with respect to unsaturated fat.

OA and LA have been found to elicit only a marginal apoptotic response at a concentration of 300 μM, but unlike SFAs, they have no effect at lower concentrations. AA (arachidonic acid; 20:4) could not induce any apoptotic response within its physiological range (1 to 10μM) in human GCs [30]. Furthermore, Valckx et al. 2014 [44] revealed that OA could reduce the expression of *BAX* and induce *BCL2* and *Gadd45b* (growth arrest and DNA damage-inducible beta) expression in human GCs. A

recent report revealed that AA could promote the survival of bovine GCs at lower concentrations (50 μM) but had detrimental effects at higher concentrations (200 μM).

OA downregulates the mRNA expression of different genes associated with FSH and LH signaling in bovine GCs such as steroidogenic acute regulatory protein (*STAR*), *CYP19A1, FSHR, LHCGR,* cytochrome P450 family 11 sub-family A member 1 (*CYP11A1*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), *CCND2* and proliferating cell nuclear antigen (*PCNA*), which have opposite effects as shown by SFAs [22]. Estrogen receptor 2 (*ESR2*), follistatin (*FST*), and *PPARG* are among the other genes that were down-regulated by OA in these cells [45]. However, the protein abundance of the above genes has yet to be determined. Similar to OA, CLA, an isomer of LA has been found to affect FSH and IGF-1 induced steroidogenesis by down-regulating the transcription of *CYP19A1*, insulin-like growth factor 1 receptor (*IGFR1*) and *GATA4* genes [46]. These observed effects were attributed to decreased levels of Akt phosphorylation and increased protein abundance of PPARG and phosphatase and tensin homolog (PTEN) by CLA in buffalo GCs [46]. It was reported that PPARG activation decreased E2 production by inducing the ubiquitination of cyclin D1 and estrogen receptor α, and that it inhibited the expression of *CYP19A1* through nuclear factor-kappaB (NFkB) activation [47, 48]. Nevertheless, decreased E2 levels by both CLA and OA suggest that UFAs are detrimental to female steroid production during metabolic stress conditions. However, the opposite regulatory effects on Akt phosphorylation by OA and CLA is very intriguing for future studies to understand the importance of Akt signaling concerning different FAs.

It seems that UFAs play an important role in PCOS pathophysiology, whose etiology is largely attributed to high levels of androgens [49]. Elevated circulatory concentrations of OA were correlated with the adverse pregnancy outcomes in obese women with PCOS undergoing controlled ovarian hyperstimulation [14]. Huang et al 2018 [50] showed that PCOS affects the metabolism of UFAs in rats. In particular, metabolites of AA generated by the cyclooxygenase enzyme were significantly increased in the ovaries of PCOS rats. These AA derived metabolites could modulate the ovarian cycle and induce luteolysis. Niu et al 2014 [14] showed that levels of OA in the follicular fluid were significantly higher in obese PCOS patients compared to obese women, indicating a role of OA in PCOS. More critical cues on the role of OA can be derived from the regulation of forkhead transcriptional regulator (FOXL2) expression. OA has been shown to have a dose dependent effect on both mRNA and protein expression of FOXL2 in FSH treated cultured bovine GCs [26] (Figure 2). FOXL2 is vital for the biogenesis of female steroids and is required to prevent the trans-differentiation of the ovary into testis, thus preventing androgen production, as shown in mice [51]. Uhlenhaut et al. 2009 [52] showed that the deletion of FOXL2 immediately increases the expression of testes-specific SRY-box transcription factor 9 (SOX9) in the mouse ovary and as a consequence, leads to comparable testosterone production as in male littermates. In agreement with this earlier report, OA supplementation promoted SOX9 expression in bovine GCs and inhibited E2 biosynthesis [45]. It has been shown that serum anti Mullerian hormone (AMH) levels were increased in patients with PCOS [53]. However, no correlation was observed with respect to circulatory NEFAs and AMH in

cows and humans [54, 55]. In any case, future experiments are needed to identify the effects of different NEFAs on AMH production by GCs.

A few reports have shown the effects of UFAs on progesterone (P4) production under serum-supplemented conditions. In goats, OA and LA, but not ALA, could induce P4 production by increasing the phosphorylation of the mitogen-activated protein kinase, ERK1/2 [56]. Supplementation with the ERK1/2 inhibitor U0126 decreased the OA and LA induced P4 production. However, these results indicate that OA and LA may induce premature luteinization of GCs. Zhang et al 2019 [57] recently reported effects of AA on bovine GCs. It is clear from their data that AA induces ERK and Akt phosphorylation similar to OA and LA. Furthermore, the expression of different marker genes such as *CYP19A1, FSHR, HSD3B1* and *STAR,* and the production of E2 were decreased by AA in a dose-dependent manner. Interestingly, AA dramatically increased the production of P4, which is quite unexpected considering the downregulation of *HSD3B1*. This indicates that the correlation of *HSD3B1* gene expression and P4 progesterone production may not be stringent at the transcriptional level [57]. On the other hand, unlike in goats, OA supplementation did not affect P4 production in bovine GCs cultured with 10% fetal calf serum (FCS) [20]. These opposite effects might be partly attributed to species differences and to the addition of serum in the culture media, whereas GCs inside the ovarian follicle have no direct contact with blood serum due to the non-vascularized GCs layer. It is well known that IGF-1 and FSH signaling plays a vital role in GCs proliferation *via* the protein kinase A (PKA) and phosphoinositide 3-kinase (PI3K) pathways. In this regard, was is reported in goats that OA could affect the IGF-1 but not the FSH-induced proliferation of GCs under serum-supplemented conditions.

Overall, unlike for SFAs, it is not completely clear whether UFA metabolites can induce apoptosis. However, multiple reports state that OA, CLA, and AA can down-regulate E2 production and perhaps also the proliferation of GCs. Furthermore, MUFAs could play a role in the etiology of PCOS as they could promote androgen biosynthesis. Studies are yet to be performed to clarify the role of other UFAs such as ALA on GC function.

4. Effects of NEFA on oocytes and early embryonic development

During follicular development, oocytes undergo an essential maturation process to become competent for fertilization. Therefore, the composition of biomolecules in the follicular microenvironment is a critical factor determining the fate of the oocyte. Several researchers have studied the effects of NEFAs on oocyte maturation and developmental competence to understand the plausible role of NEFAs in metabolic stress-induced subfertility [19, 21, 37, 42, 58, 59]. Aardema et al 2011 [42] inferred that NEFAs must be transferred through the cumulus cell layers to reach the oocyte. The transmembrane fatty acid translocase, CD36 mediates the uptake of NEFA in cumulus cells from the follicular fluid. The transport of FAs from cumulus cells to oocytes was thought to take place *via* gap junctions with the help of FA binding proteins [60]. However, FA transport into the oocyte is yet to be fully understood. Upon entering the oocyte, NEFAs are involved in the generation of lipid droplets (LDs), which are dispersed in the cytoplasm of the oocyte. LDs in oocytes may play a key role in oocyte homeostasis, as they were found to be functionally associated with the mitochondria, ER, endosomes, peroxisomes, and cytoskeleton [61, 62]. During the subsequent developmental progression of the oocyte, most LDs are degraded, and the liberated

NEFAs are utilized for essential cellular functions, including mitochondrial oxidation in the embryo [63]. Therefore, LD accumulation has been considered as a valid marker for healthy oocyte maturation in bovine and human *in vitro* fertilization (IVF) procedures [64]. However, elevated levels of NEFAs in the follicular fluid have been widely reported to cause poor COC morphology, affect oocyte maturation and decrease the numbers of cleaved embryos in humans, cows, pigs, and mice [19, 59, 65-67]. These adverse effects can also be witnessed at the DNA level as embryos generated under high NEFA exposure have altered DNA methylation and transcriptomic fingerprints of genes related to cell death and metabolic disorder [68]. Following fertilization, the presumed zygote undergoes extensive chromatin remodeling, which includes DNA methylation [69]. DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*) plays a vital role during both the growth and differentiation of mammalian oocytes, especially during maturation and early development [70]. High NEFA exposure downregulates the expression of *DNMT3A*, indicating a direct link between NEFAs and epigenetic programming of embryos [58]. Therefore, it appears that metabolic stress induced subfertility could also be due to epigenetic programming induced by NEFAs in the embryo. In the following sections, we review the major effects induced in oocytes by SFAs and UFAs in different species.

4.1 Saturated fatty acids

A growing number of publications report that SFAs have detrimental effects on oocyte quality. A recent analysis in bovine oocytes revealed that blastocysts derived after PA treatment (150 μM) were inferior in quality and had a high proportion of apoptotic cells in the inner cell mass [71]. This observation is in line with an earlier report showing that exposing bovine oocytes to high concentrations of SA resulted in a significant reduction

of the number of oocytes that reach the blastocyst stage with high expression of the maternally imprinted gene *IGF2R* [72] (Figure 3). Dysregulation of *IGF2R* perturbs placental and fetal growth [73]. Supplementing of the *in vitro* maturation medium of COCs with SFAs inhibits the cumulus expansion and progression of oocytes from metaphase II to blastocysts in bovines [74]. Later studies revealed that these FAs could further reduce the survival of bovine blastocysts under *in vitro* conditions [75]. Similar adverse effects of SFAs have been documented in ewes, where oocytes matured in the presence of 60 μM PA showed signs of impaired maturation, decreased viability, cleavage, and embryo production rates [76]. In contrast, Sinclair et al. 2008 [77] showed that increased levels of SA in the follicular fluid have been associated with the generation of morphologically favorable COCs, but increasing PA levels were associated with poor COCs bovines.

SFAs induce apoptosis by inducing mitochondrial dysfunction and ER stress in maturing oocytes [36]. PA is reported to induce significant alterations in the expression of genes related to the mitochondrial activity (cytochrome c oxidase subunit 5a (*COX5A*)) and oxidative stress (calreticulin, heat shock protein 90 kDa beta member 1 (*HSP90B1*)) of COCs, which is followed by a reduced rate of cleavage and quality of embryos 48 hrs post-insemination [71, 78]. Similarly, increased concentrations of SFAs facilitate the production of apoptotic inducers such as ceramide and reactive oxygen species (ROS) together with the altered mitochondrial membrane potential [79, 80], which can negatively affect oocyte quality and embryo development (Figure 3). Increased ROS production is associated with frequent Ca^{2+} oscillations from the ER lumen into the cytosol, as reported in H_2O_2 treated mouse oocytes, thus promoting apoptosis [81, 82].

An increased intracellular free Ca^{2+} concentration leads to the activation of Ca^{2+}-dependent proteases micro-calpain and caspase-12 in bovine preimplantation embryos [83].

Apart from altered calcium signaling, the expression of classic ER Stress markers such sas *ATF4* (activating transcription factor 4) and *HSPA5* (heat shock protein 5) is also upregulated in COCs matured in the presence of NEFA mixtures containing mainly SFAs, suggesting that protein misfolding pathways can also be disrupted by high NEFAs levels [38] (Figure 3). Similar expression patterns of ER stress markers were reported *in situ* and *in vitro* in mouse COCs exposed to lipid-rich environments, and also in COCs of obese women [24, 84, 85]. ER stress also leads to the accumulation of misfolded proteins, releasing the ER chaperone,78-kDa glucose-regulated protein (GRP78), which leads to the aggregation of ER transmembrane signaling proteins mainly PERK (protein kinase RNA-like endoplasmic reticulum kinase), IRE1 (inositol-requiring enzyme 1) and ATF6 (activating transcription factor 6) and thus commencing the UPR (unfolded protein response) [86]. PERK, C/EBP homologous protein (CHOP), and ATF4 (activating transcription factor 4) are functionally associated with each other and induce translocation of the pro-apoptotic protein BAX from the cytosol to the mitochondria and reduce the anti-apoptotic protein BCL-2 [87, 88]. Therefore, chronic ROS generation, excessive Ca^{2+} release, and high expression of proapoptotic proteins can lead to the formation of the BAX pore followed by the release of cytochrome-c and the activation of the caspase cascade in mammalian oocytes [89]. Interestingly, the lipotoxicity induced by SFAs appears to be different between the oocyte and cumulus cells, as revealed by proteomic analysis in which only 4 out of 136 regulated proteins

were found to be commonly regulated between the bovine cumulus cells and oocytes [71]. However, the majority of regulated proteins were located in the ER and mitochondria in both groups, indicating the significance of the unfolded protein response and apoptosis upon exposure to SFAs.

Overall, a majority of earlier studies are in agreement that SFAs induce ER stress and disrupt mitochondrial function, thus causing impaired developmental competence in oocytes of different species. However, there is a lack of consensus over the effects of SA on oocyte maturation and health, which can be clarified by future research.

4.2 Unsaturated fatty acids

Accumulating scientific data show that the nutritional supplementation of unsaturated fats could benefit female reproduction by improving the ovarian follicular development and oocyte developmental competence. In particular, PUFAs enriched diets are known to alleviate the NEB, which is a major detriment to female fertility and thus for the herd development in cattle and buffalo during early lactation [90]. Robinson et al. 2002 [91] reported that the size of the dominant follicle increased in cows fed LA enriched diets, whereas cows fed ALA-rich diets showed increased levels of E2 during the follicular phase. Further studies revealed that PUFAs could also improve oocyte developmental competence as the number of blastomeres in morulae increased in cows fed with flaxseed (rich in ALA) compared to those fed with saturated fat (high in PA and SA) or sunflower seed (high in LA). The above nutritional data indicate that UFAs supplementation, especially PUFAs supplementation, could help to improve oocyte development.

However, there are uncertainties in asserting the effects of other UFAs on oocyte function as several *in vitro* reports emphasize discrepancies in different types of FAs

and their corresponding effects on oocyte competence. Jorritsma et al.2004) [20] showed that treatment with UFAs such as OA delayed the maturation of COCs, decreased the fertilization and embryo cleavage rates, and had an overall negative impact on embryonic development. Supplementation with a combination of OA together with PA and SA, resulted in a similar outcome with a reduced number of oocytes reaching the blastocyst stage [72]. In contrast, Aardema et al. 2013 [7] showed that OA is quite harmless to oocytes even at higher concentrations. Another UFA, LA, has also been reported having adverse effects on bovine oocyte development. Increased concentrations of LA dramatically reduced cumulus expansion by decreasing the phosphorylation of Akt and mitogen-activated protein kinase-3 (MAPK3). LA supplementation also reduced the cyclic adenosine monophosphate (cAMP) production in bovine COCs [92]. In a subsequent paper, Marei et al. 2012 [93] reported that LA could alter the mitochondrial distribution, decrease the mitochondrial membrane potential, and increase ROS production in bovine oocytes undergoing maturation. However, these negative effects of LA were counterbalanced by the supplementation of antioxidants, such as vitamin E and glutathione peroxidase [94]. On the other hand, ALA has no such negative effects on oocyte function at physiological concentrations. Interestingly, Marei et al. 2017 [95] reported that ALA could protect bovine oocytes against the combined lipotoxic effects of elevated PA, SA and OA by improving their developmental competence through increased mitochondrial activity and reduced ER stress levels and apoptosis in bovine cumulus cells.

It has been shown that stearoyl Co-A desaturase (SCD) activity is closely associated with lipid metabolism in dairy cows and may influence their reproductive performance

[96]. SCD catalyzes the conversion of SA and PA into oleate and palmitoleate, respectively. Increased expression of stearoyl-CoA desaturase-1 (SCD1) and stearoyl-CoA desaturase-5 (SCD5) in human and SCD1 in bovine cumulus cells was correlated with improved oocyte competence [97, 98]. Importantly, SCD activity is inhibited by SA in bovine cumulus cells of intact COCs, thus causing impaired oocyte development compared to denuded oocytes exposed to SA in the absence of cumulus cells [98]. SCD inhibition in human COCs not only leads to compromised oocyte development but also to reduced aromatase expression, which is followed by decreased E2 production [99].

It appears that UFAs, particularly, OA and ALA can reduce the lipotoxicity induced by SFAs during oocyte maturation and blastocyst formation. However, OA and LA also have detrimental effects on oocyte maturation and developmental competence. No adverse impacts of ALA have been documented, and perhaps supplementing omega 3 PUFAs in the diet and during *in vitro* culture conditions could be highly beneficial to the oocyte function in comparison to other UFAs.

5. Concluding remarks:

A variety of metabolic conditions/diseases are characterized by elevated concentrations of NEFAs in the blood and eventually in the follicular fluid of humans, cows, sheep, and pigs. Based on the reviewed scientific data, it is apparent that the degree of unsaturation in the acyl carbon chain of fatty acids could determine their biological effects on GCs and oocytes. Both normal and elevated levels of SFAs, and elevated levels of MUFAs (e.g. OA) and PUFAs (e.g. LA and AA) have severe detrimental effects on the GC function and developmental competence of oocytes, thus acting as foes in

the ovarian follicle. No negative impacts of omega-3 fatty acids (ALAs) have been reported on GCs and oocytes, and perhaps they can be considered as friends in the ovarian follicle. Given the essential nature of FAs, such as LA and ALA, we think that metabolic diseases may not raise the concentration of LA and ALA in the circulation. Therefore, the levels of PA, SA and OA are the major detriments to ovarian function during metabolic diseases. The combined lipotoxicity of these three FAs can be ameliorated by ALA in GCs and oocytes. More studies are yet to be performed with respect to their intracellular signaling, especially PKA, Akt, and mitogen-activated protein kinase (MAPK) pathways, which would help to better understand their effects in cross-talk with FSH and IGF1 signalling and could help to generate novel therapeutics for subfertility.

LIST OF ABBREVIATIONS

AA: Arachidonic acid

ACO: Acyl CoA oxidase

ALA: α-linolenic acid

AMPK: 5′ adenosine monophosphate-activated protein kinase

ATF4: Activating transcription factor 4

cAMP: cyclic adenosine monophosphate

CCND2: Cyclin D2

CLA: Conjugated linoleic acid

COC: Cumulus oocyte complex

CPT1: Carnitine palmitoyl transferase-1

CYP11A1: Cytochrome P450 family 11 subfamily A member 1

CYP19A1: Cytochrome P450, family 19, subfamily A, polypeptide 1

E2: 17β-estradiol

ESR2: Estrogen receptor 2

FA: Fatty acid

FOXL2: Forkhead box protein L2

FSH: Follicle-stimulating hormone

FSHR: Follicle-stimulating hormone receptor

FST: Follistatin

GC: Granulosa cells

GnRH: Gonadotropin-releasing hormone

HDL: High-density lipoproteins

HSD3B1: 3beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type I

HSPA5: Heat shock protein family A (Hsp70) member 5

IGF-1: Insulin-like growth factor 1

LA: Linoleic acid

LDL: Low-density lipoproteins

LH: Luteinizing hormone

MAPK: mitogen-activated protein kinase

MUFA: Monounsaturated fatty acid

NEB: Negative energy balance

NEFA: Non-esterified fatty acids

OA: Oleic acid

PA: Palmitic acid

PCNA: Proliferation cell nuclear antigen

PCOS: Polycystic ovarian syndrome

PI3K: Phosphoinositide 3-kinase-3-kinase

PKA: Protein kinase A

PPAR: Peroxisomal proliferator-activated receptors

PUFA: Polyunsaturated fatty acid

SA: Stearic acid

SCD: Stearoyl coA desaturase 1

SMASE: Sphingomyelinase

SOX9: SRY (sex determining region Y)-box 9

SPT: Serine palmitoyltransferase

VLDL: Very low-density lipoproteins

DECLARATIONS:

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not Applicable

CONSENT OF PUBLICATION

Not Applicable

COMPETING INTERESTS

Authors declare no financial, non-financial and ethical conflict of interest

AUTHOR CONTRIBUTIONS

VSB and JV conceived the manuscript. VSB and AS executed review research, writing the manuscript and designed graphics. VSB finalized the draft. JV performed corrections and final edits and improved the manuscript. All authors approved the final version of the manuscript.

AVAILABILITY OF SUPPORTING DATA

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

FUNDING:

Authors thank DFG (BA 6909/1-1) and Alexander von Humboldt foundation (Ref 3.5-IND-1187133-HFST-P) for providing research funds to VSB. Authors also acknowledge the German Federal Ministry of Food and Agriculture (BMEL) (Geschäftszeichen: FITCOW 313-06.01 -28-1 -79.003-15) for supporting AS.

ACKNOWLEDGEMENTS

Not Applicable

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Figure legends:

Figure 1: Folliculogenesis: Ovarian follicles undergo a sequential development in order to release a mature oocyte for fertilization. Elevated levels of NEFAs affect the ovarian follicular development by preventing dominant follicle formation and inhibiting ovulation

Figure 2: Effect of FAs on the physiology of cultured GCs: (A) Under standard in vitro culture conditions, GCs show a typical fibroblast-like morphology in the presence of FSH and IGF1. GCs display an active expression of gonadotrophin (FSHR and LHCGR) and IGF-1 receptors whose signaling could promote steroidogenesis (*CYP19A1*, *STAR*, and *HSD3B1)* and cell proliferation (CCND2 and PCNA) *via* PKA (protein kinase A) and Akt activation. (B) Saturated fatty acids (C16:0 and C18:0) induce adverse morphological changes in GCs with an increasing number of cells undergoing

apoptosis. Decreased phosphorylation of Akt was reported in GCs. Increased expression of CD36 (fatty acid transporter), IGF-1 and FSH regulated genes can be found in GCs (C) Unsaturated fatty acids, such as OA, at elevated concentrations, also induce adverse morphological changes with increased expression of *CD36* (fatty acid transporter) leading to lipid accumulation. Increased Akt phosphorylation is hypothesized upon OA treatment. The expression of gonadotrophin receptors, steroidogenic and proliferation genes is down-regulated.

Figure 3: Effects of elevated levels of saturated FAs on oocytes: Elevated levels of saturated fatty acids induce ER stress due to the accumulation of misfolded proteins in the ER lumen, which induces the ER transmembrane signaling proteins PERK, IRE-1 and ATF-6. Induction of C/EBP homology protein (CHOP) via ATF-4 produces excessive reactive oxygen species (ROS) which leads to impaired mitochondrial membrane potential ($\Delta\Psi m$) and to the release of stored intracellular calcium ions from the ER lumen into the cytosol, eventually initiating a downstream apoptotic cascade. All these events lead to impaired oocyte maturation and developmental competence.

Table 1: Concentrations of NEFAs in different metabolic diseases in humans

Species	Condition	Biofluid	Concentration of NEFA	Ref.
Women	Obese non-diabetic	Plasma	290 µM	**[100]**
	Obese type 2 Diabetes	Plasma	621 µM *	
Human (sex not defined)	Healthy	Plasma	0.18±0.09 (SD) g/L	**[101]**
	Type 2 Diabetes	Plasma	0.45±0.21 (SD) g/L	
Women	Normal weight	Follicular Fluid	0.22 ± 0.02 (SD) mM	**[12]**
	Overweight	Follicular Fluid	0.24 ± 0.03 (SD) mM	
	Obese	Follicular Fluid	0.31 ± 0.08 (SD) mM *	
Women	18.5 ≤BMI ≤24.9 (n=60)	Serum	0.60±0.20 (SD) mM	**[16]**
	25.0 ≤BMI ≤29.9 (n=26)	Serum	0.70±0.23 (SD) mM	
	BMI ≥30.0 (n=20)	Serum	0.70±0.20 (SD) mM	
Women	Non Obese	Follicular Fluid	0.16±0.02 (SEM) meq/L	**[24]**
	Obese	Follicular Fluid	0.38± 0.04 (SEM) meq/L *	
Women	Non Obese	Plasma	0.49±0.22 (SD) mM	**[13]**
	Obese	Plasma	0.37±0.18 (SD) mM	
	PCOS Non Obese	Plasma	0.49±0.23 (SD) mM	
	PCOS Obese	Plasma	0.70±0.13 (SD) mM *	
Women	Healthy Control	Serum	4.36±2.52 (SD)mg/dl	**[15]**
	PCOS	Serum	6.93±3.51(SD) mg/dl *	
Women	Healthy fertile	Serum	3.1 ± 0.01 mg/dl	**[102]**
	Infertile	Serum	4.7 ± 0.08mg/dl *	

Women	Healthy fertile	Follicular Fluid	3.5 ± 0.03 mg/dl	**[102]**
	Infertile	Follicular Fluid	6.0± 0.1 * mg/dl	

*, significantly different levels as indicated by authors; SD, standard deviation; SEM, standard error of the mean

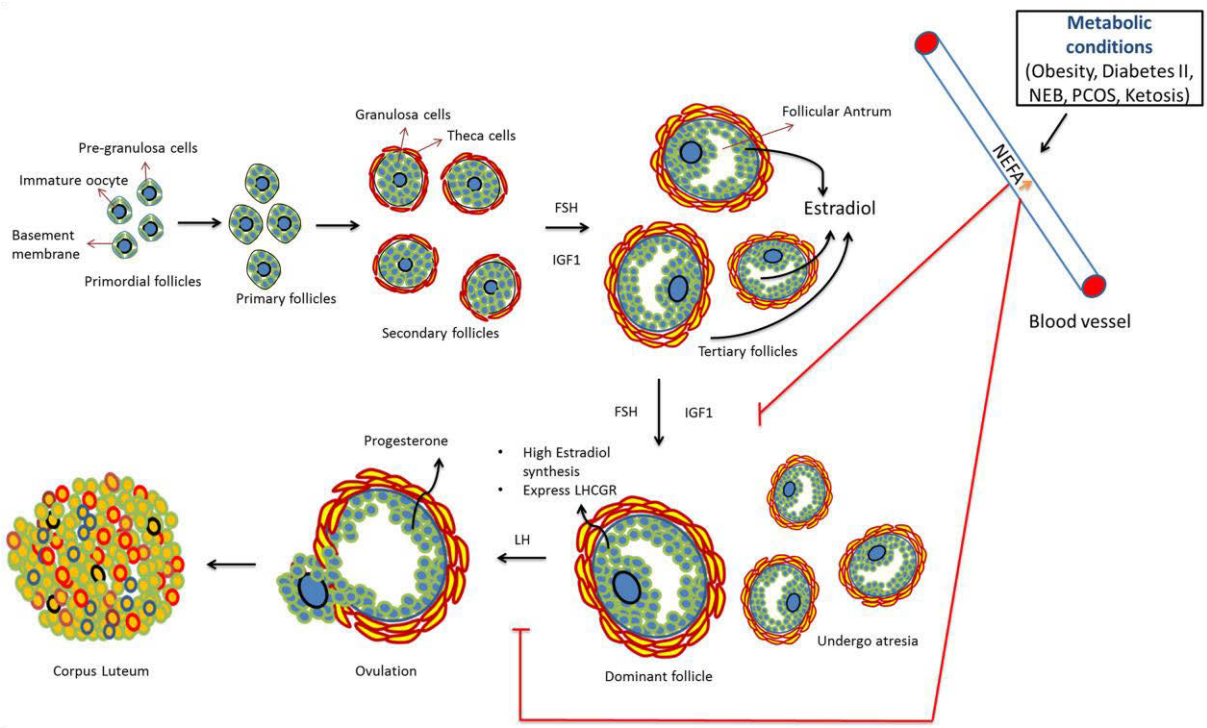
Table 2: Concentrations of NEFAs in different metabolic diseases in animals

Cows	7 days pre-parturition	Serum	~0.2 mM	**[19]**
	16 days post-parturition	Serum	0.4-1.2 mM *	
	44 days post-parturition	Serum	0.1-0.3 mM	
Cows	16 days post-parturition	Follicular Fluid	0.2-0.6 mM	**[19]**
	44 days post-parturition	Follicular Fluid	0.1-0.3 mM *	
Cows	Control	Follicular Fluid	control level	**[7]**
	Fasting (4 days)	Follicular Fluid	higher level *	
Cows	Control	Serum	control level	**[7]**
	Fasting (4 days)	Serum	higher level *	
Cows	Cycling	Plasma	0.21±0.05 mM	**[103]**
	Inactive ovary	Plasma	0.32±0.12 mM *	
Cows	Cycling cows	Blood	0.4±0.1 (SEM) mM *	**[104]**
	Cystic ovarian cows	Blood	0.7±0.1 (SEM) mM	
Ewes Pregnant	Control	Serum	0.65 mM	**[105]**
	Subclinical ketosis	Serum	1.02 mM *	
Ewes Lambed	Control	Serum	0.47 mM	
	Subclinical ketosis	Serum	0.69 mM *	
Ewes Lactating	Control	Serum	0.21 mM	
	Subclinical ketosis	Serum	0.45 ± 0.03 (SD) mM *	

Dog	Lean dog	Plasma	0.97±0.09 (SEM) mM	**[106]**
	Obese dog	Plasma	1.59±0.12 (SEM) mM	

*, significantly different levels as indicated by authors; SD, standard deviation; SEM, standard error of the mean

Figure 1


Metabolic conditions
(Obesity, Diabetes II, NEB, PCOS, Ketosis)
NEFA
Blood vessel
Pre-granulosa cells
Immature oocyte
Basement membrane
Primordial follicles
Primary follicles
Granulosa cells
Theca cells
Secondary follicles
FSH
IGF1
Follicular Antrum
Estradiol
Tertiary follicles
FSH
IGF1
Progesterone
High Estradiol synthesis
Express LHCGR
LH
Corpus Luteum
Ovulation
Dominant follicle
Undergo atresia

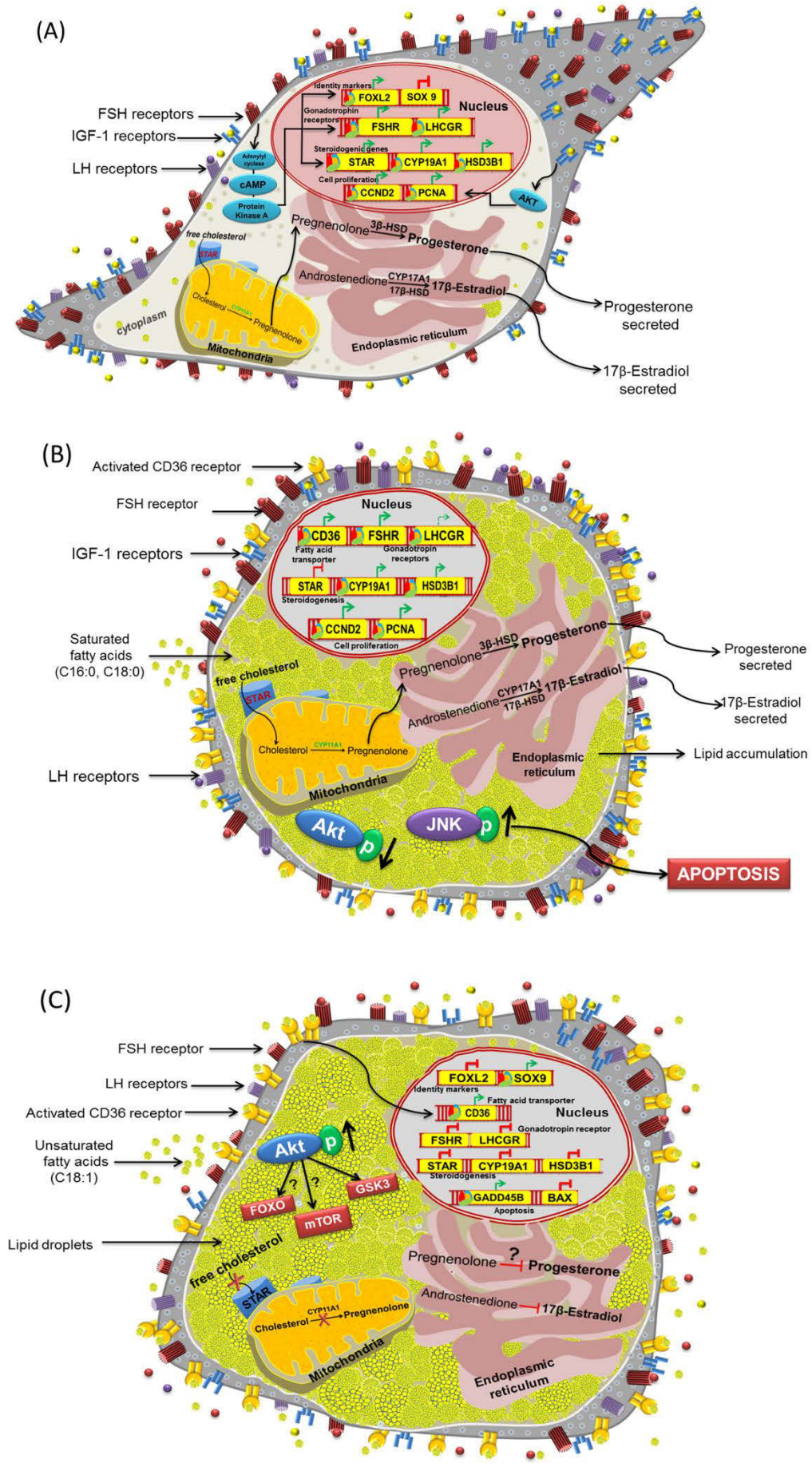
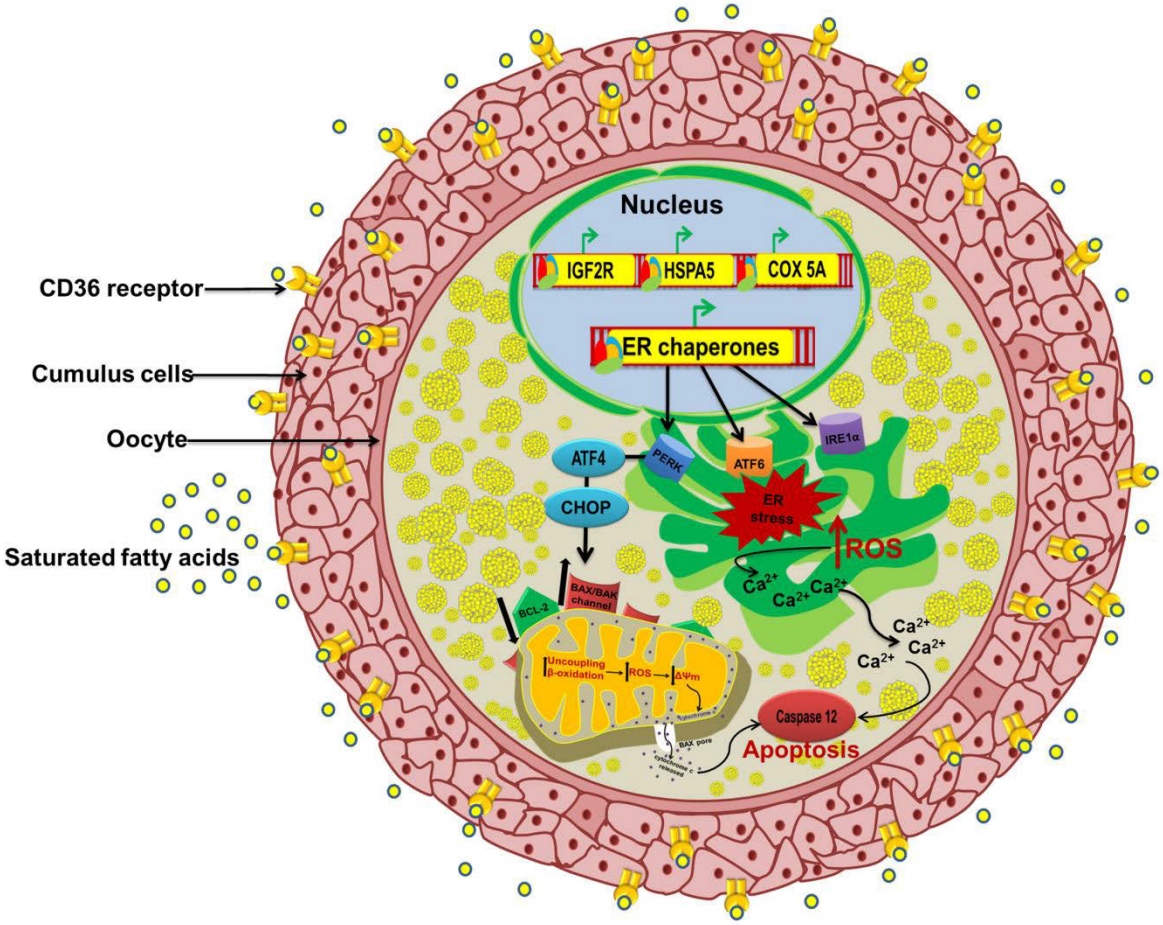

(A)
FSH receptors
IGF-1 receptors
LH receptors
Identity markers
FOXL2
SOX 9
Nucleus
Gonadotrophin receptors
FSHR
LHCGR
Steroidogenic genes
STAR
CYP19A1
HSD3B1
Cell proliferation
CCND2
PCNA
AKT
Adenylyl cyclase
cAMP
Protein Kinase A
free cholesterol
Pregnenolone
3β-HSD
Progesterone
Androstenedione
CYP17A1
17β-HSD
17β-Estradiol
Progesterone secreted
17β-Estradiol secreted
cytoplasm
Cholesterol
Pregnenolone
Mitochondria
Endoplasmic reticulum
(B)
Activated CD36 receptor
FSH receptor
IGF-1 receptors
Saturated fatty acids (C16:0, C18:0)
LH receptors
Nucleus
CD36
FSHR
LHCGR
Fatty acid transporter
Gonadotropin receptors
STAR
CYP19A1
HSD3B1
Steroidogenesis
CCND2
PCNA
Cell proliferation
free cholesterol
Pregnenolone
3β-HSD
Progesterone
Progesterone secreted
Androstenedione
CYP17A1
17β-HSD
17β-Estradiol
17β-Estradiol secreted
Cholesterol
Pregnenolone
Mitochondria
Endoplasmic reticulum
Lipid accumulation
Akt
JNK
APOPTOSIS
(C)
FSH receptor
LH receptors
Activated CD36 receptor
Unsaturated fatty acids (C18:1)
Lipid droplets
FOXL2
SOX9
Identity markers
Fatty acid transporter
CD36
Nucleus
Gonadotropin receptor
FSHR
LHCGR
STAR
CYP19A1
HSD3B1
Steroidogenesis
GADD45B
BAX
Apoptosis
Akt
GSK3
FOXO
mTOR
free cholesterol
Pregnenolone
Progesterone
Androstenedione
17β-Estradiol
Cholesterol
Pregnenolone
Mitochondria
Endoplasmic reticulum

Figure 2

Figure 3


CD36 receptor
Cumulus cells
Oocyte
Saturated fatty acids
Nucleus
IGF2R
HSPA5
COX 5A
ER chaperones
ATF4
PERK
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IRE1α
CHOP
ER stress
ROS
BAX/BAK channel
BCL-2
Uncoupling β-oxidation
ROS
ΔΨm
Caspase 12
Apoptosis
BAX pore
Ca2+

Study IV.

Low oxygen levels induce early luteinization associated changes in bovine granulosa cells

Baddela VS, Sharma A, Viergutz T, Koczan D, Vanselow J. *Frontiers in Physiology* (2018), 9:1066. (doi: 10.3389/fphys.2018.01066)

Brief summary:

The study was designed to investigate the effects of low O_2 level (1%) on bovine GCs. The production of E2 and P4 were reduced at low O_2 level compared to normal O_2 level (21%). mRNA microarray analysis revealed about 1104 differentially regulated genes of which 505 were up- and 599 were down-regulated under low O_2 level. IPA identified 36 significantly affected canonical pathways. Of these, particularly, "estrogen-mediated S-phase entry" and "cyclins and cell cycle regulation" pathway were found to be greatly down-regulated at low O_2 level. Up-regulation of key genes of angiogenesis, inflammation and glucose metabolism and down-regulation of FSH signaling, steroidogenesis and cell proliferation suggested an induction of early luteinization associated changes in GCs. Importantly, unmethylated CpG sites in the CYP19A1 promoter region suggests that GCs in low O_2 level *in vitro* were not completely transformed into luteal cells. Thus, the study demonstrates that low O_2 levels might be instrumental in initiating early events of luteinization of GCs in preovulatory follicles.

frontiers in Physiology

ORIGINAL RESEARCH
published: 07 August 2018
doi: 10.3389/fphys.2018.01066

Low Oxygen Levels Induce Early Luteinization Associated Changes in Bovine Granulosa Cells

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During follicle maturation, oxygen levels continuously decrease in the follicular fluid and reach lowest levels in the preovulatory follicle. The current study was designed to comprehensively understand effects of low oxygen levels on bovine granulosa cells (GC) using our established estrogen active GC culture model. As evident from flow cytometry analysis the viability of GC was not found to be affected at severely low oxygen condition (1% O_2) compared to normal (atmospheric) oxygen condition (21% O_2). Estimations of hormone concentrations using competitive radioimmunoassay revealed that the production of estradiol and progesterone was significantly reduced at low oxygen condition. To understand the genome-wide changes of gene expression, mRNA microarray analysis was performed using Affymetrix's Bovine Gene 1.0 ST Arrays. This resulted in the identification of 1104 differentially regulated genes of which 505 were up- and 599 down-regulated under low oxygen conditions. Pathway analysis using Ingenuity pathway analyzer (IPA) identified 36 significantly affected ($p < 0.05$) canonical pathways. Importantly, pathways like "Estrogen-mediated S-phase Entry" and "Cyclins and Cell Cycle Regulation" were found to be greatly down-regulated at low oxygen levels. This was experimentally validated using flow cytometry based cell cycle analysis. Up-regulation of critical genes associated with angiogenesis, inflammation, and glucose metabolism, and down-regulation of FSH signaling, steroidogenesis and cell proliferation indicated that low oxygen levels induced early luteinization associated changes in granulosa cells. Identification of unmethylated CpG sites in the *CYP19A1* promoter region suggests that granulosa cells were not completely transformed into luteal cells under the present low oxygen *in vitro* condition. In addition, the comparison with earlier published *in vivo* microarray data indicated that 1107 genes showed a similar expression pattern in granulosa cells at low oxygen levels (*in vitro*) as found in preovulatory follicles after the LH surge (*in vivo*). Overall, our findings demonstrate for the first time that low oxygen levels in preovulatory follicles may play an important role in supporting early events of luteinization in granulosa cells.

Keywords: granulosa cells, oxygen levels, preovulatory follicle, gene expression, luteinization

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Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *14 May 2018*
Accepted: *17 July 2018*
Published: *07 August 2018*

Citation:
Baddela VS, Sharma A, Viergutz T, Koczan D and Vanselow J (2018) Low Oxygen Levels Induce Early Luteinization Associated Changes in Bovine Granulosa Cells. Front. Physiol. 9:1066. doi: 10.3389/fphys.2018.01066

INTRODUCTION

Ovaries are the female's primary reproductive organs, which contain a large pool of primordial follicles. Under the influence of different endocrine factors, primordial follicles start maturation by forming an antrum that is filled with follicular fluid. The wall of antral ovarian follicles includes a peripheral vascularized theca cell layer which is separated from the inner avascular granulosa cell layer by a basement membrane (Siu and Cheng, 2013; Feng et al., 2017). Therefore, oxygen released from the capillaries will first reach the thecal cells. Lower amounts of oxygen will then diffuse through the basement membrane to reach the multiple layers of mural followed by antral GC and then to the cumulus – oocyte complex (COC). This clearly suggests that antral GC along with the COC are exposed to relatively low oxygen levels in large antral follicles. According to mathematic modeling, the dissolved concentrations of diffused oxygen in human ovarian follicular fluid are predicted between 11 and 51 mmHg, which corresponds to 1.5–6.7% of oxygen (Redding et al., 2008). Several other studies agree with these estimates and reported that the dissolved oxygen levels in follicular fluid is between 1 and 5% (Van Blerkom et al., 1997; Huey et al., 1999), which is far less than the atmospheric oxygen concentration (21%) that is generally used for culturing of follicular granulosa cells.

Granulosa cells are the major steroidogenic cells of ovarian follicles. During follicule maturation, follicle stimulating hormone (FSH) arouses steroid hormone production from the granulosa layer by inducing FSH receptor signaling (Rouillier et al., 1996). Once the follicle becomes dominant, an intense luteinizing hormone (LH) pulse from the pituitary gland increases the circulatory and follicular LH concentration, thus inducing early processes of luteinization and finally culminating in ovulation (Duffy and Stouffer, 2003). Dominant follicles will further increase in size during the post LH surge period, growing to a maximum diameter of 15–22 mm close to ovulation (Sartori et al., 2001; Christenson et al., 2013). As the diameter of ovarian follicles greatly increases during follicle maturation, the diffusion distance for gasses inside the follicle also increases. This will eventually lead to a continuous decrease of the oxygen concentration in the follicular fluid (Fischer et al., 1992). It has been already observed that falling pO_2 is accompanied with a decrease of pH and an increase of pCO_2 in the follicular fluid, indicating an active anaerobic respiration by follicular cells. These findings were further strengthened by the identification of increased glucose consumption and lactate accumulation in post hCG murine follicles (Harris et al., 2007). Recently, it was shown that even under normoxic conditions lactate can act as an effective signaling molecule that induces granulosa cell differentiation (Baufeld and Vanselow, 2018).

It is well known that cells of the granulosa and theca layers undergo early luteinization in preovulatory follicles immediately after the LH surge and display features that are very different from those found in dominant follicles before LH, and eventually become fully luteinized cells of the corpus luteum (Tesařík and Dvořak, 1982; Christenson et al., 2013). The most prominent changes associated with early luteinization of GC include a cease of GC proliferation, down-regulation of FSH signaling and steroidogenesis, and up-regulation of HIF1a signaling and angiogenesis (Christenson et al., 2013; Wissing et al., 2014). As the luteinization of GC commences within preovulatory follicles, we hypothesize that the prevailing low oxygen levels in preovulatory follicles may play a role in early luteinization of granulosa cells. Accordingly, the current study was carried out in our established estrogen active granulosa cell culture model to understand effects of low oxygen levels on the genome wide gene expression changes and steroid production in bovine granulosa cells.

MATERIALS AND METHODS

Culturing of Granulosa Cells

Ovaries were collected at a commercial abattoir and granulosa cells were aspirated from small to medium follicles (≤6 mm) with a syringe and 18G needles. The number of viable cells was determined using the trypan blue exclusion method and the cells were eventually cryopreserved as described in a previous manuscript (Baufeld and Vanselow, 2013). All the chemicals for cell culture were purchased from Biochrom (Berlin, Germany) unless stated otherwise. Alpha (α)-MEM was enriched with supplements to make a working medium containing 0.1% BSA, 20 mM HEPES, 0.084% sodium bicarbonate, 2 mM Glutamin, 5 μg/ml transferrin, 4 ng/ml sodium selenite, 1 mM non-essential amino acids, 10 ng/ml insulin, 100 IU penicillin, and 0.1 mg/ml streptomycin. In addition, 20 ng/ml FSH (Sigma-Aldrich, Steinheim, Germany), 50 ng/ml IGF-I (Sigma-Aldrich, Steinheim, Germany) and 2 μM androstenedione (Sigma-Aldrich, Steinheim, Germany) were added to the α-MEM just before plating the cells. For experiments, the cryopreserved cells were rapidly thawed in a water bath, washed with α-MEM and plated at a density of $\sim 1.4 \times 10^5$ viable cells per well in 24 well culture plate, which were pre-coated with collagen. The culture plates were kept in a CO_2 incubator at 21% O_2 and 5% CO_2 during the next 6 days, with media exchange every 48 h. On the 6th day, cells were incubated at low oxygen (1% O_2) and normal oxygen conditions (21% O_2), separately, for the next 48 h to analyze the low oxygen induced effects.

Cell Viability and Apoptosis Analysis

On the 8th day of culture, spent culture media were collected in a 1.5 ml collection tube and centrifuged to pellet the floating dead cells. The attached cells in the culture plate were washed twice with PBS and detached by adding 250 μl of tryplE solution (Thermo Fischer, United States) to each well of the 24 well plates. The detached cells were added to the above pelleted cells to ensure the inclusion of floating and attached cells into the analysis. The cells were pelleted and washed using 1 ml of α-MEM and subjected to viability and apoptosis analysis using the Annexin-V FITC kit (Miltenyi Biotec, Germany). Briefly, the cell pellet was re-suspended in 100 μl of 1× binding buffer followed by adding 10 μl of Annexin V reagent. After gentle mixing, the tubes were incubated in the dark for 15 min. Cells were washed and re-suspended in 500 μl of 1× binding buffer. Then 5 μl of propidium iodide (PI) was added to the cells and mixed

gently just before the flow cytometry analysis. The fluorescence signal was quantified from single cells (10,000 counts) using a flow cytometer (Gallios, Beckman-Coulter, Germany) and the data were analyzed using the Kaluza-software (Beckman-Coulter, Germany).

RNA Isolation, cDNA Preparation, qPCR Analysis

Total RNA was isolated using the Nucleo Spin RNA II Kit (Macherey-Nagel, Düren, Germany) by following the manufacturer's instructions. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Bonn, Germany) and cDNA was prepared using the SensiFast cDNA synthesis kit (Bioline, Luckenwalde, Germany). The gene expression analysis for the selected genes was performed using the SensiFast SYBR No-ROX (Bioline) reagent and gene specific primers (**Supplementary Data Sheet S1**) in a Light Cycler 96 instrument (Roche, Mannheim, Germany). For qPCR analysis, two different volumes (2 and 4 μl) of cDNA were amplified in 12 μl total reaction volume. The qPCR cycling conditions used are shown in **Supplementary Data Sheet S2**. Amplicons from all analyzed genes, were cloned in PGEM-T vectors (Promega Biosciences, United States) and sequenced to verify the product. External standard curves were generated during each run from plasmids at five different serially diluted concentrations (5×10^{-12} to 5×10^{-16} g plasmid). The Abundance of transcripts was normalized using TBP as a validated house-keeping gene under low oxygen conditions (Baddela et al., 2014).

Microarray Analysis

To identify changes of the global gene expression profiles induced by low oxygen levels, total RNA was isolated from GC cultured under normal (NOL 1–4) and low oxygen levels (LOL 1–4) and analyzed using GeneChip™ Bovine Gene 1.0 ST Array (Affymetrix®, Inc., Santa Clara, CA, United States). RNA integrity was measured in a bio analyzer instrument, which revealed RIN values from 9.7 to 10 in all samples (**Supplementary PDF File S1**). The subsequent amplification and labeling of RNA samples was performed using GeneChip 3′ amplification and one-cycle target labeling reagents. Overnight hybridization of RNA samples and probes was carried out in a hybridization oven followed by acquisition of the gene expression signals using an Affymetrix Gene Chip Scanner 3000. Normalization and background reduction of gene expression was performed using the robust multichip average method. The acquired data were subsequently analyzed using the TAC 4.0 software (Transcriptome Analysis console 4.0, Affymetrix). Differentially expressed (DE) genes were recognized using the cut off parameters, fold difference $>|2|$, ANOVA $p < 0.05$, and FDR (q) < 0.05.

Estimation of Estradiol and Progesterone Concentration

Estradiol (E2) and progesterone (P4) concentrations were estimated in the spent media using a sensitive single antibody ^{3}H-radioimmunoassay performed in a competitive mode. The antibodies were raised in rabbit and purified using affinity chromatography. The E2 tracer, (2,4,6,7-3H estradiol-17β), was purchased from GE Healthcare (Freiburg, Germany) and P4 tracer, [1,2,6,7-3H(N) progesterone], was purchased from PerkinElmer (Boston, United States). Assay standards were prepared in RIA buffer after dissolving the tracers in 100% ethanol. The levels of radioactivity were measured in a liquid scintillation beta-counter containing an integrated RIA-calculation program (TriCarb 2900 TR; PerkinElmer, Germany).

Determination of Cell Proliferation

Cell proliferation was analyzed by identifying the number of cells in different phases of the cell cycle using flow cytometer analysis. GC were cultured for 8 days as described above. On the 8th day, spent culture media were collected in a 1.5 ml collection tube and centrifuged to pellet floating cells. The cells were washed twice using 1× PBS and then detached from the plate by adding 250 μl of tryplE reagent (Thermo Fisher, United States) to each well. The detached cells were added to the floating cells of the corresponding wells. Cells were pelleted, washed and dissolved in 300 μl of 1× PBS. The cell suspension was subsequently added dropwise into 70% ice cold ethanol and stored at −20°C for 2 h. Then, cells were centrifuged (300 × *g*, 10 min, 4°C), re-suspended in 1 ml RNase solution (1 mg/ml) and incubated at 37°C for 30 min. The PI reagent (final concentration 50 μg/ml) was added to the cells and incubated for 30 min at 37°C in the dark. The fluorescence was quantified from single cells (10,000 counts) using a flow cytometer (EPICS-XL, Beckman-Coulter, Krefeld, Germany). The data were subsequently analyzed using the Multicycle software (Phoenix, United States).

Methylation Analysis of CpG Sites in *CYP19A1* Promoter 2.0

Methylation of *CYP19A1* at three CpG dinucleotide positions −35, +18, and +30, relative to the GC-specific start site of transcription, in the proximal promoter 2.0 region were analyzed using the bisulfite direct sequencing method. Genomic DNA was isolated from GC cultured under normal oxygen (n = 5) and low oxygen (n = 5) conditions and modified using the EZ DNA Methylation-Gold kit (Zymo, Freiburg, Germany). PCR was performed using HotStarTaq Plus reagents (Qiagen, Hilden, Germany) and gene specific primers (**Supplementary Data Sheet S1**) at following cycling conditions: pre-incubation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 75 s, annealing at 53°C for 75 s, and extension at 72°C for 35 s. PCR products were analyzed by agarose gel electrophoresis (3%, ethidium bromide stained) and purified using the High Pure PCR Purification Kit (Roche). Sequencing of PCR products was performed at the institutional core facility. The sequence files were evaluated using a Web based software QUMA (QUantification tool for Methylation Analysis), available at http://quma.cdb.riken.jp/top/index.html, to quantify the percent of methylated vs. un-methylated cytosine nucleotides at individual CpG dinucleotides.

Bioinformatics and Statistical Analysis

All bioinformatic analyses were carried out for the human homologs of DE genes. The enriched gene ontology terms were

recognized using WebGestalt, a WEB based gene set analysis tool kit. The canonical pathways and upstream regulators were identified using the Ingenuity pathway analysis tool (IPA, Qiagen, Hilden). Further, hub genes were recognized by constructing a protein-protein interaction network using NetworkAnalyst tool available at www.Networkanalyst.ca. Microarray data analysis was performed using integrated statistical measures available in TAC 4.0 software. Analysis of Variance (ANOVA) was used to calculate the *p*-values, which were further corrected by False Discovery Rate (Benjamini–Hochberg method) measures. Significance levels were set at fold change > |2|, ANOVA $p < 0.05$, and FDR (q) < 0.05 for identifying DE genes. The qPCR gene expression, RIA and flow cytometry values were analyzed by using *t*-test in GraphPad prism 5.0 software. Significant changes were acknowledged if $p < 0.05$.

RESULTS

Effect of Low Oxygen Levels on the Viability and Steroidogenesis of Granulosa Cells

After subjecting GC to low and normal oxygen levels (**Figure 1** and **Supplementary Figure S1**), the percentage of live, apoptotic and dead cells was determined using flow cytometric analysis by adding propidium iodide (PI) and annexin-V reagents to the detached cells. This revealed that GC did not show significant variation in healthy viable (PI−, Annexin−), apoptotic (PI−, Annexin+) and dead (PI+, Annexin+) cell counts at low oxygen levels compared to cells grown at normal oxygen levels (**Figure 1C**). However, unlike the viability status of the cells, levels of estradiol and progesterone were significantly reduced at low oxygen levels (**Figure 1D**).

Microarray Data

Raw microarray data files were analyzed using the TAC 4.0 software. Evaluation of data from 3′ and 5′ hybridization controls (**Supplementary Figure S2**) and normalized signal box plots (**Supplementary Figure S3**) indicated that all array files were normal and passed the quality checkup (QC). Subsequent principal component analysis (PCA) of the microarray data sets (**Figure 2A**) showed that the samples from low and normal oxygen treatments were located most distant from each other with a variation of 77.2% on principal component axis 1 (PCA1), thus indicating the remarkable differences of the gene expression profiles between the two treatments. Further, a mere variation of 6.3 and 3.9% was observed on the PCA2 and PCA3 axes, respectively, which is mainly due the variation between samples treated with low or normal oxygen concentrations.

A total of 20422 publicly annotated gene clusters (**Supplementary Data Sheet S3**) were identified in the microarray data. Among them, 1104 genes were recognized to be differentially expressed (FC > | 2| ; $p < 0.05$ and FDR < 0.05) between the GC cultured at low and normal oxygen levels (**Supplementary Data Sheet S4**). Specifically, 505 and 599 genes were up- and down-regulated, respectively, at low oxygen levels. The abundance of DE genes was visualized in the form of an interactive heat map (with zoom in and out features), which was constructed using shinyHeatmaply package in R studio (**Supplementary Html File S1**). The same heat map is shown in a static form in **Figure 2B**. The top twenty down- and up-regulated genes at low oxygen levels are listed in **Tables 1**, **2**.

Particularly, *TXNIP* (thioredoxin interacting protein) showed strongest down-regulation (FC = 11.06; q = 4.12E-05), whereas *HBA*, encoding hemoglobin alpha chain, showed strongest up-regulation (FC = 239.21; q = 3.04E-11) under low oxygen conditions. Other important changes comprised down-regulation of granulosa cell marker genes, *FOXL2* (FC = 3.48; q = 1.08E-06), *FSHR* (FC = 3.17; q = 8.66E-08) and *CYP19A1* (FC = 7.56; q = 6.21E-09) and up-regulation of genes associated with angiogenesis, *VEGFA* (FC = 3.37; q = 3.46E-08), *VEGFB* (FC = −2.21; q = 1.91E-06), *VCAM1* (FC = −3.91; q = 2.93E-06), *EDNRA* (FC = −2.1; q = 0.0001), *ANGPT2* (FC = −5.63; q = 2.96E-07) and *ANGPTL4* (FC = −4.86; q = 5.90E-08), and inflammation related genes, *PTGES* (FC = −5.23; q = 6.12E-08), *VNN2* (FC = −4.14; q = 1.50E-07) and *VNN1* (FC = −14.97; q = 3.80E-09). Further on, multiple uncharacterized genes (*LOC104976005*, *LOC783613*, *LOC527388*, *LOC786781358,* and *LOC104968446* etc.) were found to be regulated by differential oxygen concentrations. However, their functions with respect to granulosa and luteal cell function is not yet known.

Microarray data were validated using RNA samples isolated from three independent experiments, different from those used for microarray analysis. A total of six genes were selected for re-assessing the expression values. These include, up-regulated (*VNN2* and *VEGFA*), down-regulated (*FSHR* and *CYP19A1*) and un-regulated genes (*NR5A2* and *OXT*). The normalized expression of all these genes was found to be similar in both qPCR and microarray estimations (**Figure 3**).

Bioinformatics Interpretations

All bioinformatic analyses were performed for the human homologs of DE genes. Initially, GO terms were generated separately for up- and down-regulated genes using the WebGestalt tool (**Figure 4**). This indicated the prioritized list of biological processes, cell components and molecular function categories for genes regulated by differential oxygen levels. For Example: 192 and 71 up-regulated genes are involved in "cell communication" and "cell proliferation," respectively, at LOL compared to 162 and 78 genes (down-regulated at LOL) at NOL, respectively. Likewise, genes localized in the nucleus are prioritized at LOL whereas membrane proteins are prioritized at NOL. There were 21 genes identified to have extracellular matrix functions at LOL compared to eight genes at NOL. Similar observations can also be noticed with respect to different molecular functions at different oxygen levels. Further, to understand the detailed functional changes induced by differential oxygen concentrations, enriched canonical pathways were identified using IPA. Among 283 different canonical pathways identified by IPA (**Supplementary Data Sheet S5**), 36 were significantly enriched ($p < 0.05$) and among them 17 were showing either positive or negative z score values (**Table 3**), which

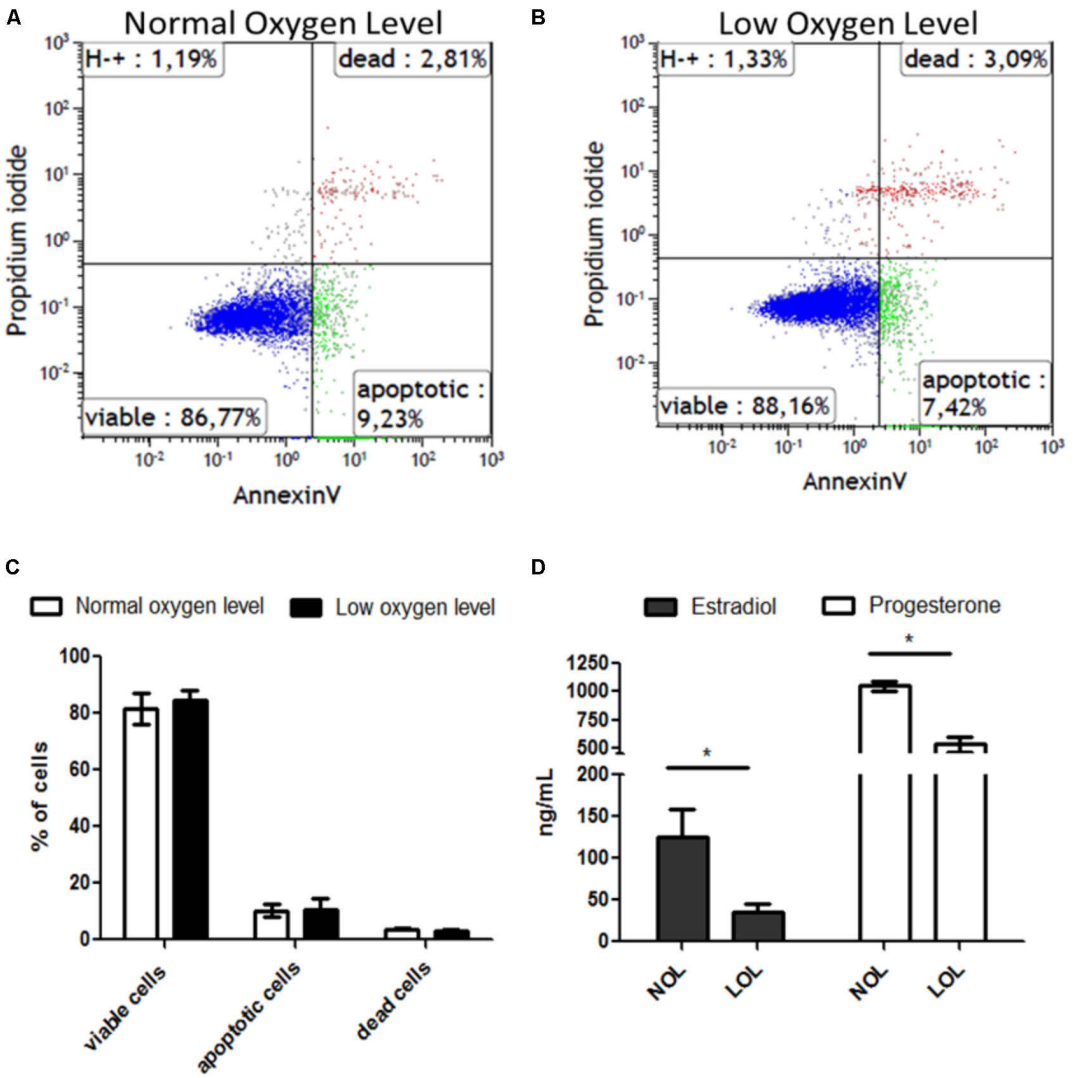

FIGURE 1 | Effect of low oxygen levels on the viability and steroidogenic capacity of granulosa cells. **(A,B)** Visualize representative histograms of cells treated with normal and low oxygen levels, respectively, in flow cytometry analysis. **(C)** Means ± SEM of three independent experiments are represented. **(D)** Estradiol (black bars) and progesterone (white bars) concentrations are shown at low (LOL) and normal oxygen levels (NOL). Results are means ± SEM of three independent experiments. Significant changes were acknowledged with asterisks if $p < 0.05$ in *t*-testing.

indicate the propensity of a particular pathway under given treatments.

Importantly, pathways associated with cellular proliferation including Estrogen-mediated S-phase Entry (p = 8.70964E-06), Cyclins and Cell Cycle regulation (p = 0.000457088), Cell Cycle: G2/M DNA Damage Checkpoint Regulation (p = 0.001778279), and Cell Cycle: G1/S Checkpoint Regulation (p = 0.004677351) were strongly affected by the oxygen concentration in granulosa cells.

Further, a zero order PPI network was identified among the DE genes to recognize the critical hub genes affected by the oxygen concentration. The resultant network contained 414 nodes with 874 connecting edges (**Figure 5** and **Supplementary Data Sheet S6**). The hub genes were ranked based on their interacting degree and betweenness in the PPI network. Importantly, *ESR1* was identified to be the most highly ranked hub gene with a degree = 70 and betweenness = 20183. It is followed by *KIAA0101* with a degree = 65 and betweenness = 16119. The top 20 ranked hub genes in the PPI network along with their degree and betweenness of interaction were mentioned in the **Table 4**.

Low Oxygen Levels Affect Cellular Proliferation

Ingenuity pathway analyzer analysis revealed that pathways related to the cell cycle were found to be majorly affected under low oxygen concentration. To validate this finding, GC were analyzed in a flow cytometer to determine the percent of cells in different stages of the cell cycle. Confirming the IPA analysis, flow cytometry analysis showed that GC were significantly

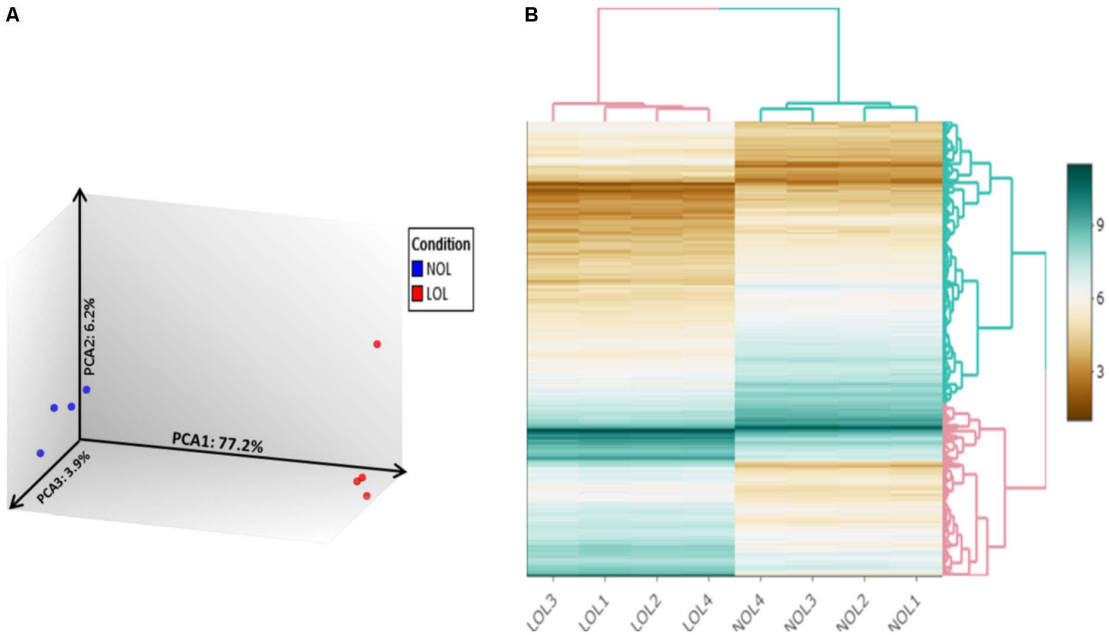

FIGURE 2 | Principle component analysis and clustered heat mapping. Unsupervised principal component analysis visualizes differences in the transcriptomes of bovine GC cultured at normal and low oxygen conditions **(A)**. Blue and red color dots denote individual samples from normal and low oxygen conditions, respectively. Each axis indicates the fraction of percentage of variation out of total mapped variation among samples. **(B)** Hierarchically clustered heatmap of differentially expressed genes between samples treated with normal (NOL1–NOL4) and low oxygen levels (LOL1–LOL4).

TABLE 1 | Top twenty down-regulated genes at low oxic conditions.

Affymetrix ID	Gene symbol	Description	MES at NOL	MES at LOL	FC (NOL/LOL)
12837074	*TXNIP*	Thioredoxin interacting protein	190.01	17.14	11.06
12836232	*LRP8*	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	369.64	45.25	8.18
12688063	*CYP19A1*	Cytochrome P450, family 19, subfamily A, polypeptide 1	413.00	54.56	7.56
12849517	*GPR85*	G protein-coupled receptor 85	25.45	3.55	7.15
12818766	*LOC104976005*	Uncharacterized LOC104976005	108.38	16.56	6.53
12786691	*LOC783613*	Dynein light chain 1, cytoplasmic	32	5.31	6.02
12802575	*LOC527388*	Histone H4	93.05	16.11	5.77
12704737	*RRM2*	Ribonucleotide reductase M2	59.71	10.33	5.75
12846849	*LOC786781; CTH*	Cystathionine gamma-lyase-like; cystathionine gamma-lyase	55.33	9.78	5.64
12774048	*RCC2; MIR2358*	Regulator of chromosome condensation 2; microRNA mir-2358	49.52	9.18	5.39
12901592	*CENPW*	Centromere protein W	9.31	1.85	5.01
12800906	*LOC104968446*	Histone H2A type 1	445.72	89.26	4.98
12697409	*ARHGAP11A*	Rho GTPase activating protein 11A	33.12	6.72	4.92
12904800	*GPR50*	G protein-coupled receptor 50	148.05	30.27	4.89
12882714	*CTH*	Cystathionine gamma-lyase	55.33	11.47	4.84
12902815	*RGN*	Regucalcin	92.41	19.15	4.82
12715265	*SLC2A10*	Solute carrier family 2 (facilitated glucose transporter), member 10	83.86	17.63	4.77
12869943	*CCNA2*	Cyclin A2	74.02	16	4.65
12735060	*SRM*	Spermidine synthase	160.89	35.75	4.5
12871633	*ELOVL6*	ELOVL fatty acid elongase 6	352.13	79.34	4.42

MES, mean expression signal; FC, fold change.

TABLE 2 | Top twenty up-regulated genes at low oxygen conditions.

Affymetrix ID	Gene symbol	Description	MES at NOL	MES at LOL	FC (NOL/LOL)
12812382	*HBA; HBA1*	Hemoglobin, alpha 2; hemoglobin, alpha 1	11.39	2721.14	−239.21
12862249	*BHLHE41*	Basic helix-loop-helix family, member e41	53.07	1144.10	−21.58
12822964	*PPP1R3C*	Protein phosphatase 1, regulatory subunit 3C	13.54	272.47	−20.04
12846870	*CIART*	Circadian associated repressor of transcription	15.56	261.37	−16.81
12900682	*VNN1*	vanin 1	20.25	302.33	−14.97
12776671	*LIMS2*	LIM and senescent cell antigen-like domains 2	22.47	312.99	−13.9
12894529	*LOXL2*	Lysyl oxidase-like 2	33.82	436.54	−12.94
12733712	*BDNF*	Brain-derived neurotrophic factor	11.63	141.04	−12.15
12864766	*KRT18*	Keratin 18	43.11	522.75	−12.09
12721948	*NDRG1*	N-myc downstream regulated 1	153.27	1833.01	−11.96
12798624	*PARP3*	Poly (ADP-ribose) polymerase family, member 3	18.76	219.79	−11.67
12850003	*FGL2*	Fibrinogen-like 2	12.99	139.10	−10.71
12766049	*NLGN2*	Neuroligin 2	45.88	477.71	−10.39
12723335	*CALB1*	Calbindin 1, 28kDa	7.31	74.54	−10.14
12889942	*ZNF395*	Zinc finger protein 395	44.63	430.53	−9.65
12727781	*TAGLN*	Transgelin	46.20	418.76	−9.09
12837477	*ACKR3*	Atypical chemokine receptor 3	35.01	298.17	−8.52
12881035	*PRR7*	Proline rich 7 (synaptic)	19.97	166.57	−8.32
12741966	*CABP1*	Calcium binding protein 1	7.16	58.08	−8.11
12903629	*RAB33A*	RAB33A, member RAS oncogene family	16.44	133.43	−8.09

MES, mean expression signal; FC, fold change.

arrested in G0/G1 phase with significantly less numbers of cells undergoing cellular proliferation at LOL (**Figures 6A,B**). Specifically, 96.68 ± SEM 0.3% of GC underwent the cell cycle arrest in the G0/G1 phase, which further ceases the cellular replication as only 1.6 ± SEM 0.1% of cells were found to be in the S phase of the cell cycle at LOL (**Figure 6C**). Whereas 91.13 ± SEM 1.5% and 6.4 ± SEM 1.6% of cells were found to be in the G0/G1 phase and S-Phase of the cell cycle, respectively, at normal oxygen conditions. No significant differences were observed in the number of cells at sub G0/G1 and G2/M phases at low and normal oxygen treatments. Further, qPCR analysis of proliferation markers, CCND2 (**Figure 6D**) and PCNA (**Figure 6E**), showed that low oxygen levels significantly down-regulated the expression of these marker transcripts, which in turn supports the data of flow cytometer and IPA analysis.

Methylation Analysis of the *CYP19A1* Proximal Promoter P 2.0

The present microarray and qPCR data revealed that *CYP19A1* expression is down-regulated in GC cultured at low oxygen conditions. It has been shown that the *CYP19A1* gene is methylated at three CpG sites that are present in the proximal promoter region at positions −35, +18 and +30 in granulosa derived luteal cells *in vivo* (Vanselow et al., 2005, 2010). Therefore, these individual CpG nucleotides were analyzed as a marker for complete luteinization in cultured GC at low and normal oxygen levels. Sequencing of bisulfite modified DNA revealed that these CpG sites are completely unmethylated (100%) in all 10 candidate DNA samples, isolated from independently cultured granulosa cells. The corresponding sequence of the *CYP19A1* proximal promoter and chromatogram of sequenced but modified DNA are shown in **Figure 7**.

DISCUSSION

Ovarian follicles are highly dynamic structures, which undergo sequential maturation, ovulation and luteinization processes during a successful reproductive cycle. Due to the lack of direct blood supply, pO_2 in the follicular fluid is found to decrease during follicular maturation, reaching lowest levels in preovulatory follicles (Fischer et al., 1992). Increased accumulation of lactate and of the hypoxia inducible factor 1a (HIF1a) in the follicular fluid additionally indicate the existence of low oxygen conditions in ovarian follicles (Harris et al., 2007; Duncan et al., 2008; Kim et al., 2009). In the present study, the observed absence of significant changes in the viability and apoptotic status of GC at LOL compared to NOL intuitively indicated that GC might be naturally adapted to survive under low oxygen levels.

However, contrasting observations were reported in other cell types in which apoptosis was induced by low oxygen levels (Zheng et al., 2012). Further, by analyzing the mRNA transcriptome, we could show that low oxygen levels alter the gene expression profile of granulosa cells in a highly specific manner.

Low Oxygen Levels Induce Early Luteinization Associated Changes of the Transcriptome in Granulosa Cells

A clear separation of samples treated with low and normal oxygen levels on PCA1 of principal component analysis visibly

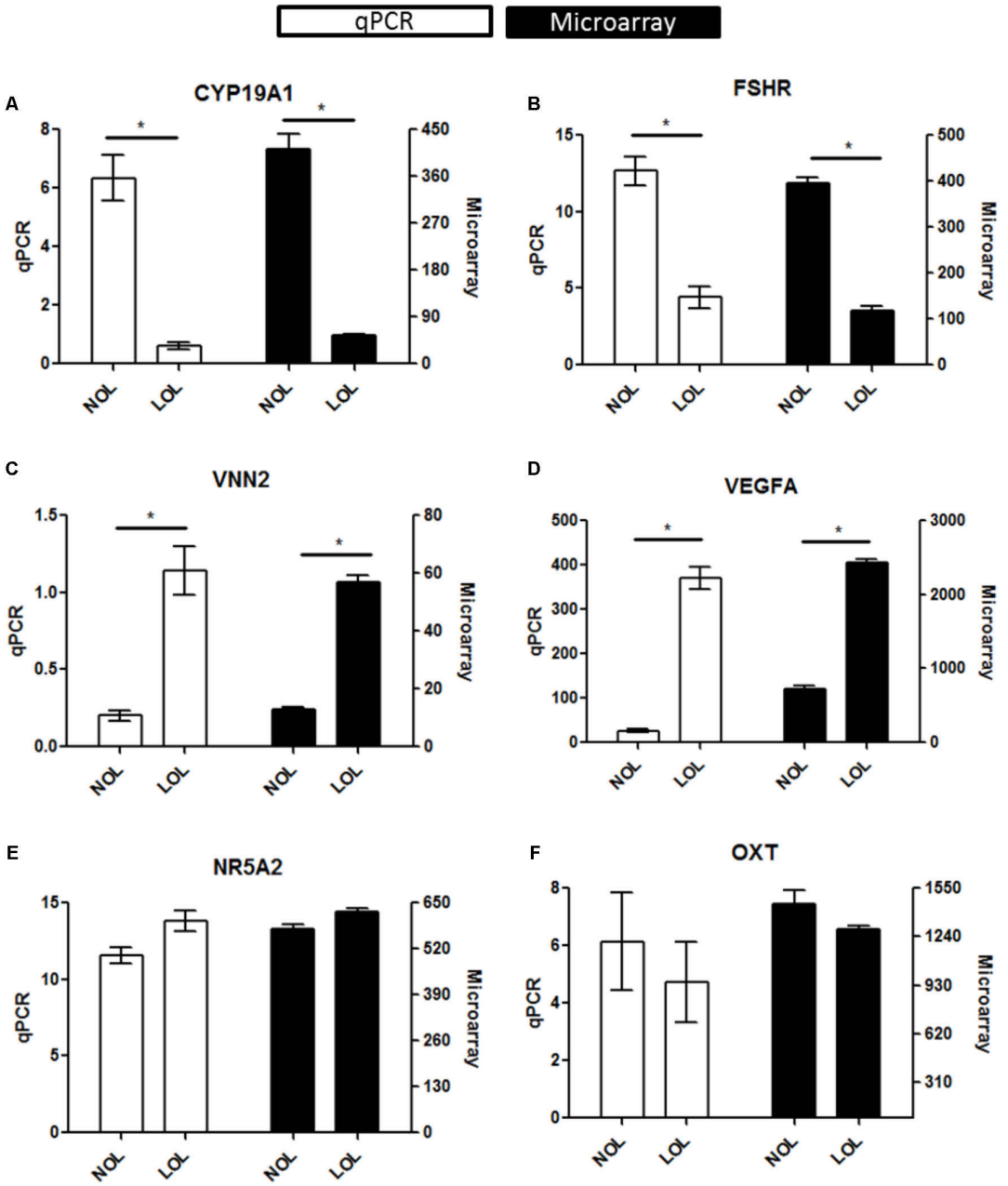

FIGURE 3 | qPCR evaluation of microarray data: Diagrams **(A–F)** represent data of selected marker genes *CYP19A1*, *FSHR*, *VNN2*, *VEGFA*, *NR5A2*, and *OXT*. The left vertical axis indicates the qPCR gene expression values (Mean ± SEM), whereas the right vertical axis indicates the linear hybridization signal values from microarray data. On the X-axis, NOL indicates normal oxygen and LOL low oxygen level condition. Significant changes (*) were acknowledged if $p < 0.05$ for qPCR values in *t*-testing. Significance was attributed to microarray signals if the Fold change > |2|, ANOVA $p < 0.05$, and FDR $q < 0.05$.

indicated that the oxygen concentration could remarkably affect the global gene expression profile in granulosa cells. The subsequent data analysis resulted in the identification of 1104 differentially expressed genes under low oxygen concentrations. Importantly, genes associated with FSH signaling, which include *FSHR*, *CYP19A1,* and *LHCGR* were significantly down-regulated at low oxygen levels. FSH signaling is a characteristic phenotypic feature of granulosa cells (Mihm and Evans, 2008). It induces the expression of *CYP19A1*, which in turn is involved in the production of estradiol from the theca layer derived androstenedione (Parakh et al., 2006). The remarkable down-regulation of *CYP19A1* expression at low oxygen level was further reflected by reduced estradiol production. Our group has earlier reported that low-level *CYP19A1* expression in granulosa lutein cells of mature CL coincides with methylation of the main ovarian *CYP19A1*

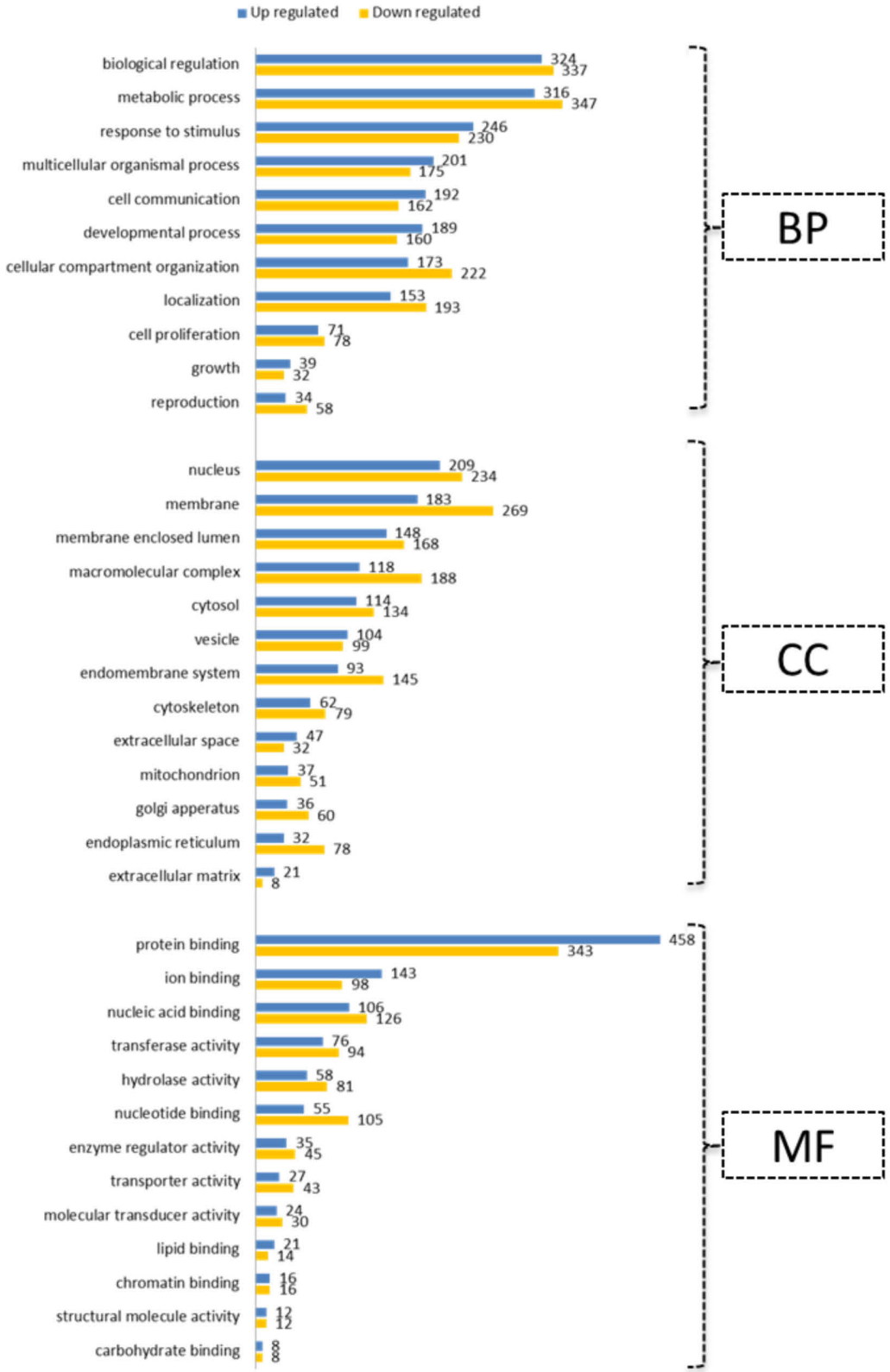

FIGURE 4 | Gene ontology annotations. Up- and down-regulated genes under low oxygen conditions were subjected to GO slim annotations. The submitted genes were classified into categories of 11 biological processes (BP), of 13 cell components (CC) and of 13 molecular functions (MF). Vertical axes indicate prioritized functions and horizontal axes indicate the number of genes associated with each of the represented annotations.

TABLE 3 | List of canonical pathways enriched for differentially expressed genes in IPA.

Ingenuity canonical pathways	*P*-value	*z*-score	Molecules
Estrogen-mediated S-phase entry	8.71E-06	3	CCNA2/E2F4/CCNE2/CDKN1A/CDKN1B/ESR1/ CDK1/CDC25A/SKP2
Mitotic roles of polo-like kinase	0.000135	2.53	KIF23/PLK4/CDC20/TGFB1/PTTG1/PRC1/CDC16/ CDK1/KIF11/CCNB1/CDC25A/SMC1A
Cyclins and cell cycle regulation	0.000457	3.317	CCNA2/E2F4/CCNE2/CCND2/TGFB1/HDAC11/ CDKN1A/TGFB2/CDKN1B/CDK1/CCNB1/SKP2/CDC25A
Aryl hydrocarbon receptor signaling	0.000537	3.051	GSTA3/CCNE2/NQO1/APAF1/CYP1B1/CHEK1/ CCNA2/JUN/CCND2/TGFB1/RARA/NEDD8/ CDKN1A/TGFB2/HSPB7/DHFR/CDKN1B/ESR1
Cell cycle: G2/M DNA damage checkpoint regulation	0.001778	−2.333	TOP2B/GADD45A/CDKN1A/TOP2A/BORA/ CDK1/CHEK1/CCNB1/SKP2
Retinoic acid Mediated apoptosis signaling	0.003162	0.378	TIPARP/RARA/ZC3HAV1/APAF1/CYCS/ PARP3/CRABP2/TNFRSF10A
Cell cycle: G1/S checkpoint regulation	0.004677	−1.89	E2F4/CCNE2/CCND2/TGFB1/HDAC11/ CDKN1A/TGFB2/CDKN1B/CDC25A/SKP2
p53 signaling	0.004898	−1.265	PIK3R3/PCNA/CCND2/JUN/GADD45A/THBS1/PIK3CG/ PIK3R1/CDKN1A/PIAS1/APAF1/TNFRSF10A/BIRC5/CHEK1
AMPK signaling	0.006761	−0.728	PFKFB3/RAB27A/CPT1A/SLC2A1/RAB3A/PIK3R1/CPT1B/ PFKL/PFKP/EIF4EBP1/PIK3R3/CCNA2/ GYS1/PFKFB4/FASN/PIK3CG/CDKN1A/PRKAA2/AK4/ ACACA/HMGCR/PHF10
Interferon signaling	0.010233	2.449	SOCS1/OAS1/PTPN2/MX1/PIAS1/TAP1
ATM signaling	0.012882	−0.302	JUN/SMC2/GADD45A/FANCD2/H2AFX/CDKN1A/ZEB1/CDK1/ CHEK1/CCNB1/CDC25A/SMC1A
Type I diabetes mellitus signaling	0.0302	1	TRAF6/SOCS1/SOCS3/PIAS1/HLA-B/APAF1/CYCS/ SOCS4/HLA-DQB1/IL1R1/MAP3K5
SAPK/JNK signaling	0.034674	0.302	MAP4K2/PIK3R3/MAP3K9/JUN/GADD45A/PIK3CG/ MAP3K13/PIK3R1/DUSP10/MAP3K5/MAP3K2
CD27 signaling in lymphocytes	0.040738	−1.633	MAP3K9/JUN/MAP3K13/APAF1/ CYCS/MAP3K5/MAP3K2
Death receptor signaling	0.041687	0.632	ACTA2/TIPARP/ZC3HAV1/APAF1/CYCS/ HSPB7/ACTG2/PARP3/MAP3K5/TNFRSF10A
Role of CHK proteins in cell cycle checkpoint control	0.044668	−0.447	PCNA/E2F4/CDKN1A/CDK1/ CHEK1/RAD1/CDC25A
NRF2-mediated oxidative stress response	0.046774	−0.816	GSTA3/PIK3R1/NQO1/DNAJC3/DNAJC10/JUNB/MAP3K5/MAFF/ PIK3R3/HMOX1/JUN/ACTA2/DNAJB11/PIK3CG/ACTG2/ FKBP5/HACD3

promoter P 2.0 (Vanselow et al., 2006, 2010). Therefore we analyzed the methylation levels of individual CpG dinucleotides within the *CYP19A1* promoter P 2.0. The present data, however, indicated that hypoxic culture conditions could not induce increased DNA-methylation levels in GC-specific *CYP19A1* promotor 2.0. This could be possibly due to the fact that the cultured GC were not completely transformed into luteal cells under low oxygen conditions.

It is well known that *FOXL2* (Forkhead box protein L2) plays an important role in upholding GC identity (Georges et al., 2014). Genomic deletion of *FOXL2* leads to the development of seminiferous tubules like structure in female mice (Uhlenhaut et al., 2009). *FOXL2* levels were found to be down-regulated in preovulatory granulosa cells and in the corpus luteum (Pisarska et al., 2004). During our study we found down-regulation of *FOXL2* under hypoxic condition in GC thus suggesting that the cells are driven toward luteinization by low oxygen levels.

Contrary to FSH signaling, GC cultured at LOL showed a significant up-regulation of multiple genes (*VEGFA*, *TGFB2*, *VCAM1*, *VEGFB*, *ENDRA*, *ANGPT2,* and *ANGPTL4*) involved in angiogenesis and endothelial cell migration. Angiogenesis is essential for the development of a functional corpus luteum (Reynolds et al., 2000), which is one of the most vascularized tissues in the body. Therefore, luteinization of GC should be accompanied by the up-regulation of the genes involved in angiogenic processes. By IPA analysis, HIF1a was identified to be the major upstream regulator of gene expression at LOL (**Supplementary Data Sheet S7**). HIF1a is a transcription factor, which is known to be up-regulated during luteinization in granulosa cells (Nishimura and Okuda, 2010). Upon binding within promotor regions, HIF1a induces the expression of different target genes that cumulatively induce the vascularization of tissues. Accordingly, "angiogenesis" ($p = 1.03E-06$) and "vasculogenesis" ($p = 6.86E-07$) were identified as up-regulated biological functions at LOL as revealed by IPA

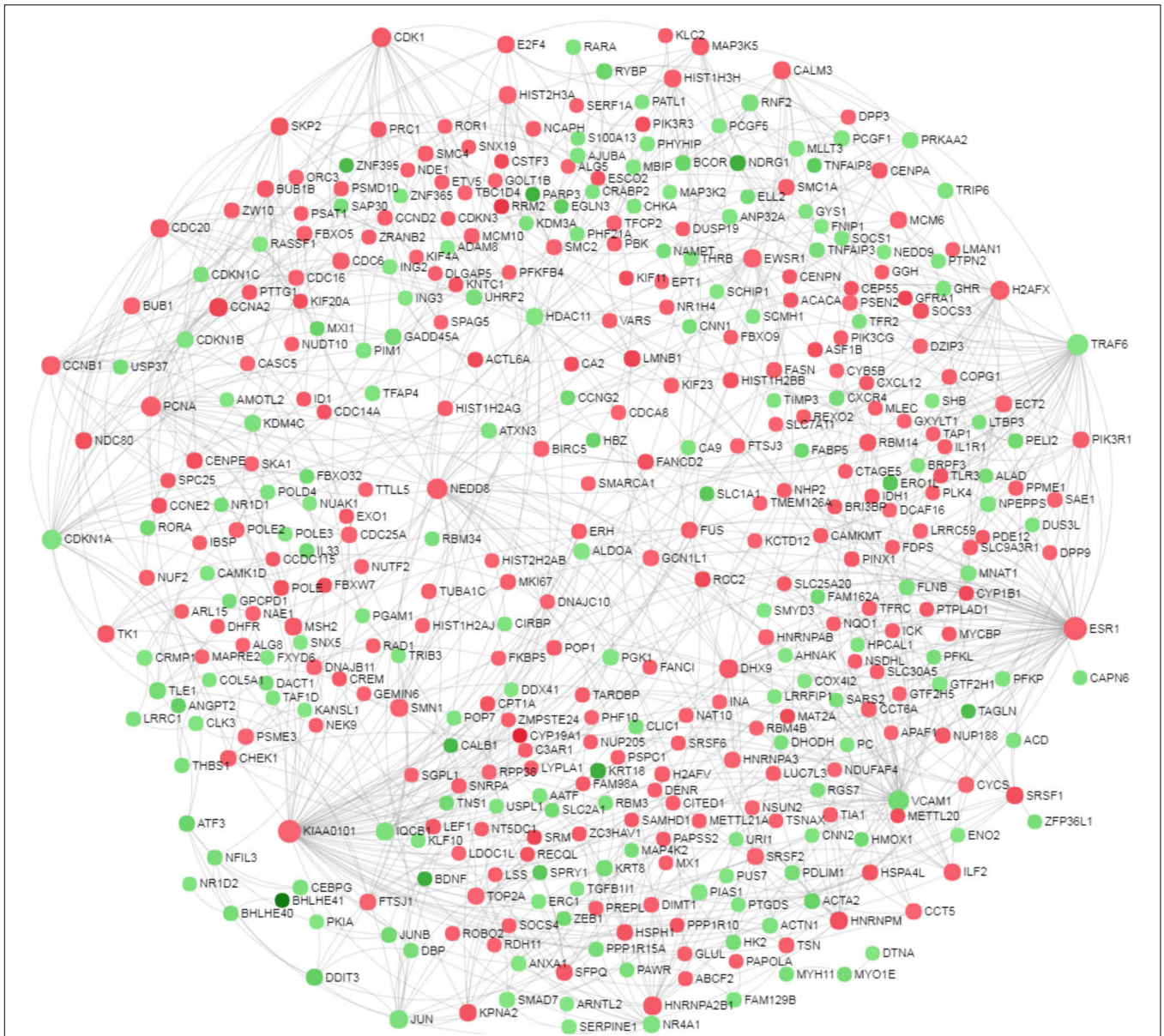

FIGURE 5 | Network based meta-analysis of differentially expressed genes. A zero order protein-protein interaction network was identified among the differentially expressed genes of the microarray data to recognize the critical hub genes. The nods with green and red color indicate down- and up-regulated transcripts under low oxygen conditions.

(**Supplementary Data Sheet S8**). HIF1a is also known to induce the glucose metabolism in different cell types (Marin-Hernandez et al., 2009). Increased glucose uptake and its metabolism have been observed in cultured murine follicles after hCG administration (Harris et al., 2007). Similarly in the present study, low oxygen levels induced the expression of *GLUT1* (Glucose transporter 1) and *GLUT 3* (Glucose transporter 3), which mediate the glucose uptake into cells. Increased expression of *GLUT1* was found in early luteal cells in response to low oxygen levels (3% O2), however, mid luteal cells failed to show such a response (Nishimura et al., 2017). In addition, substantial down-regulation of *TXNIP* expression further confirms the increased glucose metabolism at LOL. *TXNIP* is a redox sensitive signaling molecule involved in glucose metabolism (Parikh et al., 2007). Decreased expression of *TXNIP* was disclosed to be associated with increased glucose uptake in muscle and liver cells (Chutkow et al., 2008).

HBA, which encodes hemoglobin A, is majorly produced by erythrocytes. It constitutes a part of the tetrameric blood gas carrier, hemoglobin. Only a few non-hematopoietic cells are known to synthesize these proteins (Thompson et al., 2015). Interestingly, erythrocyte free HBA was identified in large antral follicles of hCG treated ovarian follicles (Thompson et al., 2015). The *HBA* gene showed a substantial up-regulation in granulosa

TABLE 4 | List of hub genes.

Gene symbol	Description	Degree	Betweenness	FC (NOL/LOL)
ESR1	Estrogen receptor 1	70	20183.19	2.19
KIAA0101	KIAA0101 ortholog	65	16118.99	2
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase	42	14937.06	−2.15
VCAM1	Vascular cell adhesion molecule 1	38	7215.39	−3.91
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8	35	7411.9	2.25
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	28	8569.35	−2.38
PCNA	Proliferating cell nuclear antigen	28	6574.45	2.35
CDK1	Cyclin-dependent kinase 1	25	5767.09	2.72
H2AFX	H2A histone family, member X	22	3622.03	2.51
DHX9	DEAH (Asp-Glu-Ala-His) box helicase 9	19	3123.61	2.72
CCNB1	cyclin B1	19	3076.66	2.3
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	18	3934.21	2.62
JUN	Jun proto-oncogene	17	5189.28	−2.88
CCNA2	Cyclin A2	16	2692.33	4.65
EWSR1	EWS RNA-binding protein 1	16	2686.22	2
CDC20	Cell division cycle 20	16	1854.56	2.74
CALM3	Calmodulin 3 (phosphorylase kinase, delta)	14	4101.26	2.28
IQCB1	IQ motif containing B1	14	1982.65	−2.88
SMN2	Survival of motor neuron 2, centromeric	14	1913.5	2.01
HNRNPM	Heterogeneous nuclear ribonucleoprotein M	14	1362.04	3.46

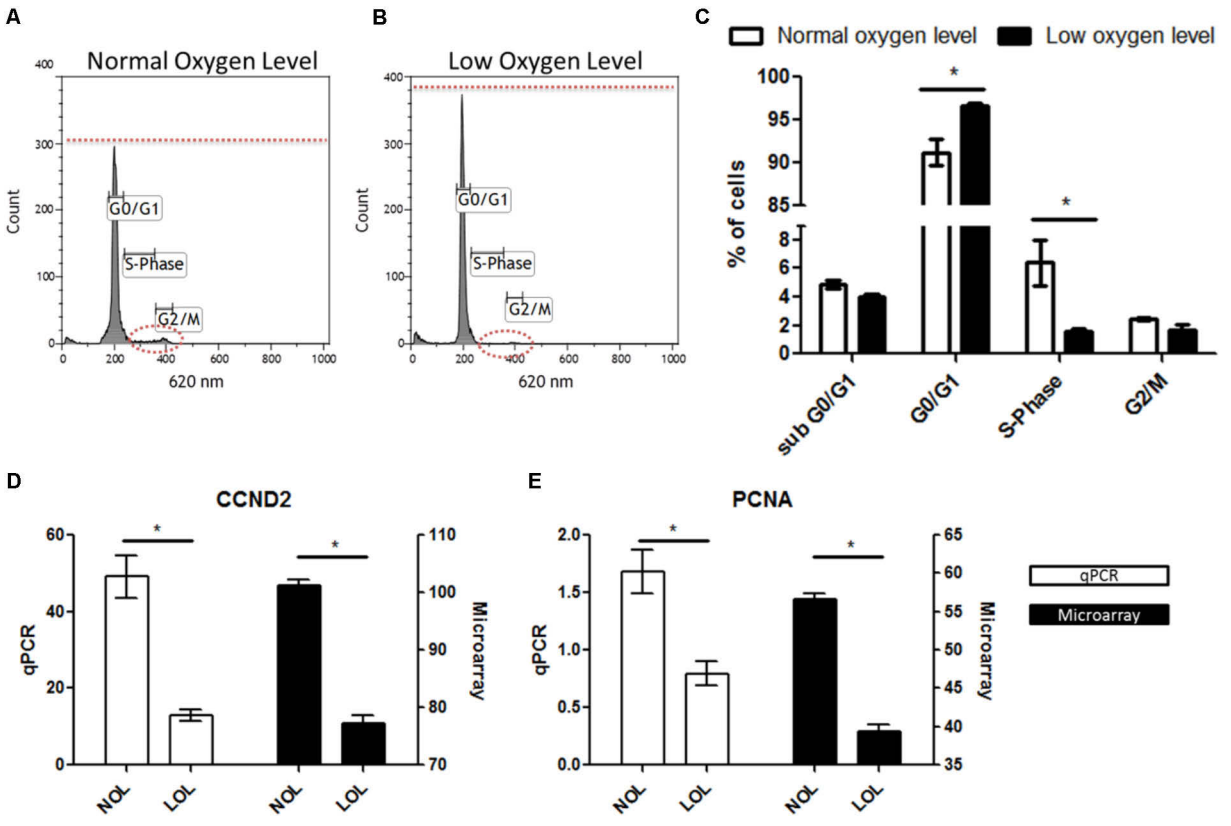

FIGURE 6 | Effects of low oxygen levels on granulosa cell proliferation. **(A,B)** Represent flow cytometry histograms of PI stained granulosa cells after normal and low oxygen level treatments, respectively. Differences in the relative fluorescence at G0/G1, S and G2/M phases were marked with dotted lines. **(C)** Mean ± SEM values of three independent experiments showing numbers of cells in different phases of the cell cycle. **(D,E)** Transcript abundance of *CCND2* and *PCNA* in granulosa cell at normal and low oxygen conditions. Significant differences (asterisks) were acknowledged when $p < 0.05$ in *t*-testing.

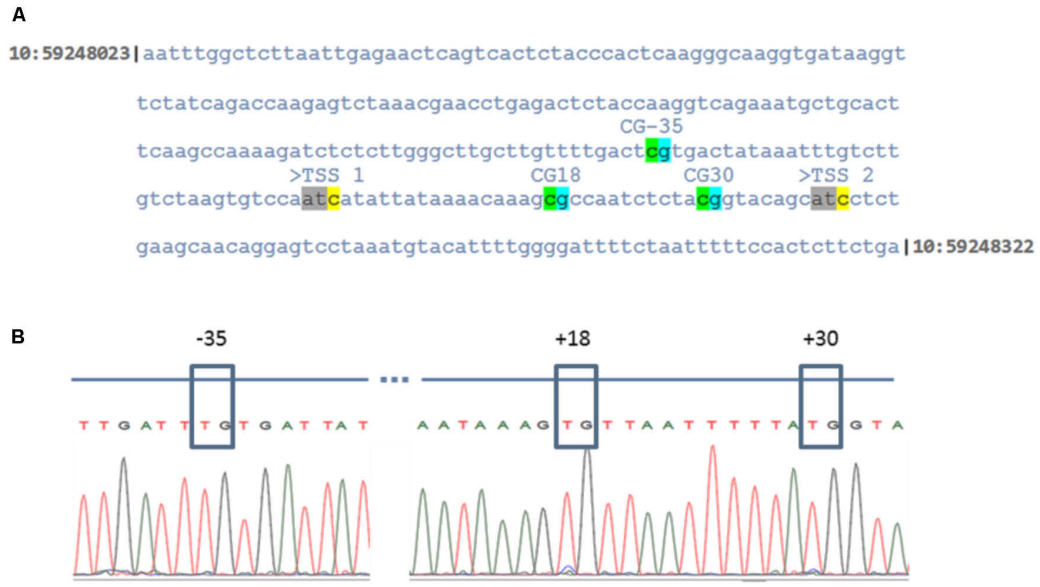

FIGURE 7 | DNA methylation levels of the proximal *CYP19A1* promoter region in granulosa cells at low and normoxic conditions. **(A)** Indicates the sequence of genomic DNA corresponding to the *CYP19A1* proximal promoter region on chromosome 10 between the genomic region, 59248023 to 59248322. The three CpG sites with respect to the main ovarian transcription start site (TSS 2) are highlighted. **(B)** Electropherogram of the bisulfite treated and sequenced *CYP19A1* promoter region. The CpG sites were boxed to show that cytosine residues were unmethylated in all samples irrespective of differential oxygen treatment.

cells cultured at LOL in the present study. Although the specific roles of hemoglobin in ovarian follicles is not yet known, identification of HBA in GC cultured at LOL further strengthens that LOL do exist in large antral follicles.

Among other important features, induction of genes associates with inflammation and leucocyte migration is widely observed in granulosa cells of preovulatory follicles (Espey, 2006; Sayasith et al., 2013). Up-regulation of such genes (*VNN1*, *VNN2*, *C1QTNF3*, *TNFAIP3* and *TNFAIP8* and *PTGES*) will further suggest that GC were on the verge to early luteinization and preceding ovulation at low oxygen conditions. However, the observation of no significant differences in the expression of *PTGS2*, one of the marker genes of post LH response, indicates that *PTGS2* might be regulated by LH but not by hypoxia in preovulatory granulosa cells.

A previous transcriptome analysis study describing effects of the preovulatory LH surge on antral bovine granulosa cells (*in vivo*) identified differential regulation of 2266 annotated genes upon LH surge (Christenson et al., 2013). Comparison of these genes with the present transcriptome data revealed that 1007 genes were similarly regulated in both studies but with different fold enrichment values (**Supplementary Data Sheet S9**). This important observation further strengthens the idea that prevailing low oxygen levels in preovulatory follicles could play an important role in inducing early molecular events in granulosa cells to prepare these cells for the formation of a functional corpus luteum.

Progesterone production was found to be decreased in GC under hypoxic conditions. Normally, progesterone biosynthesis is controlled by "LH receptor" (LHCGR) and finally synthesized by "hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1" (HSD3B1) in granulosa cells. Down regulation of these two genes was reported in GC immediately following the LH surge (Christenson et al., 2013). A similar down regulation of these two genes was observed in LOL treated GC during the present study. But, in spite of the down regulation of LHCGR and HSD3B1 expression, progesterone production was not significantly changed *in vivo* in early preovulatory ovarian follicles after LH surge (Christenson et al., 2013). In any case it needs additional studies to understand the cumulative effects of LH and hypoxia on progesterone production for further understanding of the complex *in vivo* situation.

Cellular Proliferation Is Affected at Low Oxygen Conditions

Transformation of granulosa cells into early luteal cells is associated with decreased cellular proliferation in order to promote cellular differentiation (Stocco et al., 2007; Christenson et al., 2013; Wissing et al., 2014). Pathway analysis using IPA revealed that cellular proliferation is majorly affected at low oxygen levels. Earlier reports in humans, bovine and mice showed that expression of cell proliferation markers, *PCNA* and *CCND2*, were strongly down-regulated in follicular (preovulatory) granulosa cells that are isolated at post LH surge stages (Nimz et al., 2009; Christenson et al., 2013; Wissing et al., 2014). Similarly, LOL induced a similar phenotype in cultured GC as pathways like "Estrogen-mediated S-phase Entry," "Cyclins and Cell Cycle Regulation" and "Cell Cycle: G2/M DNA Damage Checkpoint Regulation" were found to be significantly affected. Additionally, cell cycle inhibitors including *CDKN1A*, *CDKN1B,* and *CDKN1C* were also up-regulated at

LOL, which is further in line with the data of Wissing et al. (2014). Down-regulation of granulosa cell proliferation was also reflected by the PPI network in which the top hub genes are *ESR1* and *KIAA0101*, both known to be involved in regulating cell proliferation in different cell types. *ESR1* encodes for the cytosolic estrogen receptor, which upon binding estrogens, translocates into the nucleus and directly binds to the DNA at estrogen responsive elements. This results in the expression of multiple target genes including genes of cell cycle progression (Oviedo et al., 2011). Therefore down-regulation of *ESR1* expression could be one major reason for the down-regulation of the estrogen mediated S-phase entry at low oxygen conditions, as revealed by IPA. Similarly *KIAA0101* is known for its strong association with *PCNA* (Emanuele et al., 2011), which plays an important role in cell proliferation.

In summary, estrogen active granulosa cells were found to remain healthy under severely low oxygen conditions and showed specific genome wide alterations associated with down-regulation of FSH signaling, cell proliferation and steroidogenesis beside up-regulation of angiogenesis, glucose metabolism and inflammatory processes.

CONCLUSION

Based on the present data, we conclude that prevailing low oxygen levels in preovulatory follicles could play a key role in supporting luteinization of granulosa cells.

DATA DEPOSITION

The microarray data were deposited in GEO by following MIAME guidelines and can be accessed with accession number GSE112070.

AUTHOR CONTRIBUTIONS

VB and JV designed the study. VB, AS, DK, and TV executed the experiments. VB wrote the manuscript and analyzed the data. All authors read and approved the manuscript.

FUNDING

Authors also thank Alexander von Humboldt foundation for providing a fellowship/grant to VB.

ACKNOWLEDGMENTS

Authors appreciate Maren Anders, Veronika Schreiter, Christian Plinski, and Swanhild Rodewald for technical assistance during the experiments. A special thanks to Dr. Sergio Eliseo Palma Vera for his help in generating interacting heat map.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01066/full#supplementary-material

FIGURE S1 | Light microscopy pictures of cultured granulosa cells after normal and low oxygen treatments.

FIGURE S2 | Signals from the 3′ and 5′ Hybridization controls in all microarray samples.

FIGURE S3 | Signal box plots of all microarray samples.

DATA SHEET S1 | Primers.

DATA SHEET S2 | qPCR programme.

DATA SHEET S3 | Total list of annotated genes and their expression values.

DATA SHEET S4 | Differentially expressed genes along their expression values.

DATA SHEET S5 | List of canonical pathways.

DATA SHEET S6 | List of hub genes and their interactions.

DATA SHEET S7 | List of upstream regulators.

DATA SHEET S8 | List of biological functions.

DATA SHEET S9 | List of similarly regulated genes between "post LH surge follicular granulosa cells (*in vivo*)" and "low oxygen level treated granulosa cells (*in vitro*)".

HTML FILE S1 | Interactive heat map of differentially expressed genes.

PDF FILE S1 | RNA bio analyzer report.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Study V.

HIF1 driven transcriptional activity regulates steroidogenesis and proliferation of bovine granulosa cells

Baddela V.S, Sharma A, Michaelis M, Vanselow J. *Scientific Reports* (2020), 10:3906. (doi:10.1038/s41598-020-60935-1)

Brief summary:

In this study, HIF1 driven transcriptional activity in bovine GCs was investigated using echinomycin and gene knockdown procedures to suppress the HIF1 activity. Suppression of HIF1 function revealed that HIF1 at the transcription level regulates key genes of steroidogenesis (*STAR*, *HSD3B* and *CYP19A1*) and of proliferation, *CCND2* and *PCNA* in GCs. Additionally, it was shown that that *CYP19A1* is one of the possible downstream target of HIF1 in bovine GCs as indicated by gene expression, radioimmunoassay and ChIP analysis. Overall, the data suggest that HIF1 driven transcriptional activity plays a vital role in steroidogenesis and proliferation of GCs in developing ovarian follicles.

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HIF1 driven transcriptional activity regulates steroidogenesis and proliferation of bovine granulosa cells

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Hypoxia-inducible factor 1 (HIF1) is a heterodimeric transcription factor, consisting of a constitutively expressed β-subunit (HIF1B) and a regulated α-subunit (HIF1A). In the present study, we analyzed the HIF1 driven transcriptional activity in bovine granulosa cells (GC). Treatment of GC with FSH (follicle stimulating hormone) and IGF1 (insulin-like growth factor 1) resulted in the upregulation of *HIF1A* mRNA expression under normoxia. Immunohistochemistry of bovine ovarian sections showed distinct staining of HIF1A in the GC layer of different staged ovarian follicles. Suppression of HIF1 using echinomycin and gene knockdown procedures revealed that HIF1 transcriptionally regulates the genes associated with steroidogenesis (*STAR*, *HSD3B* and *CYP19A1*) and proliferation (*CCND2* and *PCNA*) of GC. Further, our data suggest that *CYP19A1*, the key gene of estradiol production, is one of the plausible downstream targets of HIF1 in bovine GC as shown by gene expression, radioimmunoassay, and chromatin precipitation analysis. Based on these results, we propose that HIF1 driven transcriptional activity plays a crucial role in GC functionality, especially steroidogenesis and proliferation in developing bovine ovarian follicles.

Hypoxia-inducible factor 1 is a pleiotropic transcription factor made up of hypoxia-inducible factor 1 A (HIF1A) and hypoxia-inducible factor 1B (HIF1B). HIF1A is the specific and regulatory constituent of HIF1 protein complex, whereas HIF1B is the constitutive subunit and forms quaternary structures with multiple other proteins such as ligand-bound aryl hydrocarbon receptor, aryl hydrocarbon receptor repressor, and HIF2A[1]. Therefore regulation of *HIF1A* expression is unique and vital to the activity of the HIF1 protein complex. HIF1 binds to hypoxia-responsive elements (HRE) present in the promoter region of target genes thus controlling their transcription. The groundbreaking investigations have revealed that HIF1 is a master transcriptional regulator of cellular response to low oxygen levels[2–4]. However, *HIF1A* is also induced and stabilized in hypoxia independent manner by different growth factors such as angiotensin II[5], prostaglandins[6], interferon-alpha[7], insulin-like growth factor 1 (IGF1)[8] etc. It has been implied that Wnt induced phosphatidylinositol 3-kinase (PI3k)/Akt signaling and signal transducers and activators of transcription 3 (STAT3), and c-Myc pathways can induce the activity of HIF1 in a hypoxia independent manner[9].

Emerging studies indicate that HIF1 is a significant regulator of gene expression in ovarian compartments, and play a role in healthy follicle development. Transcriptome analysis in pigs indicated that *HIF1A* expression is downregulated in atretic follicles compared to medium sized healthy antral follicles[10]. Expression of *HIF1A* was reported in granulosa cells (GC) of different species including human[11], mice[12], rat[13], pigs[14] and cows[15]. Kim *et al.* (2009) have shown that HIF1 is essential for ovulation process as inhibition of HIF1 activity using echinomycin, a competitive inhibitor of HIF1, caused an anovulatory phenotype in mice[12].

HIF1 regulates multiple genes in GC and contributes to the ovarian follicle development and differentiation. Zhang *et al.* (2015) have shown that knockdown of *HIF1A* mRNA abundance downregulates the *PCNA* (proliferator cell nuclear antigen) mRNA expression under normoxic conditions in rat primary GC[13], similar to renal medullary interstitial cells of rats[5]. Alam *et al.* (2004) reported that FSH (follicle stimulating hormone) mediated

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upregulation of genes involved in follicle differentiation such as *VEGFA* (vascular endothelial growth factor A), *LHCGR* (Luteinizing Hormone/Choriogonadotropin Receptor) and *INHBA* (inhibin-α) is dependent on HIF1 activity in rats[16]. Another gene, *END2* (endothelin 2), which is suggested to play a role in ovulation and luteinization processes, was found to be regulated by HIF1 in transformed human GC[17]. Similarly, *STAR* coding for steroidogenic acute regulatory protein was transcriptionally regulated by HIF1 both under normoxic and hypoxic conditions in KK1 cells, which are immortalized mouse GC[18].

It is well known that vascularization of the ovarian follicle is limited to the thecal cell layer, which is separated from the GC and cumulus-oocyte complex (COC) by a basement membrane. Therefore, it has been implied that significantly lower amounts of oxygen will be available to the intrafollicular cells as the follicle's size increases[19]. Hence, analyzing the role of HIF1 under normoxic and hypoxic environments in the presence of FSH and IGF1 would offer important cues regarding GC physiology. Accordingly, the present investigation was carried out to identify HIF1 dependent transcriptional activity both under normoxic and hypoxic conditions using our renowned estrogen active culture model of bovine primary GC[20–23].

Results

Expression of HIF1A in bovine granulosa cells. The effect of FSH was analyzed at three different concentrations, such as 2 ng/ml, 10 ng/ml and 20 ng/ml (Fig. 1a). At 2 and 10 ng ml FSH, the expression of *HIF1A* was not altered in GC. However, *HIF1A* was significantly induced at 20 ng/ml FSH compared to the control group (0 ng FSH and 0 ng IGF1). Likewise, the effect of IGF1 was analyzed at concentrations of 2 ng/ml, 25 ng/ml and 50 ng/ml (Fig. 1a). Similar to FSH, IGF1 was unable to induce *HIF1A* at the lowest concentration. However, the expression of *HIF1A* was profoundly increased at 25 ng/ml and 50 ng/ml. No difference in the *HIF1A* expression was observed between 25 ng/ml and 50 ng/ml IGF1 treatments. The western probing analysis showed that FSH (20 ng/ml) and IGF1 (50 ng/ml) supplemented GC synthesize HIF1A protein under normoxia (Fig. 1b). Immunohistochemistry of bovine ovarian follicles revealed that HIF1A proteins are distinctly expressed in the GC layer of primary, secondary, tertiary, and large antral follicles, which are in general under the influence of FSH and IGF1 *in-vivo* (Fig. 1c).

Effects of echinomycin on granulosa cells. Initially, effect of echinomycin on cell GC viability was analyzed to primarily to avoid cytotoxic doses of echinomycin in GC cultures. Results showed that 5 nM of echinomycin has no significant effect on cell viability, apoptosis and dead cell status compared to the control cells treated with 20 ng/ml FSH and 50 ng/ml IGF1 without echinomycin (Supplementary Information: S3,S4). In subsequent analyses, the expression of *HIF1A* (Fig. 2a) was decreased under normoxic and hypoxic conditions upon echinomycin treatment. Echinomycin significantly downregulated the expression of *VEGFA* (Fig. 2b), which is a well-known downstream target of HIF1 in many cell types, including GC. Therefore, the decrease in *VEGFA* expression reflects the successful inhibition of HIF1 activity by echinomycin both under normoxic and hypoxic conditions. On the contrary, inhibition of HIF1 function caused an increased expression of an inflammatory gene, vascular non-inflammatory molecule 2 (*VNN2*) (Fig. 2c).

Steroidogenesis. Echinomycin did not affect the *STAR* and *HSD3B* (3 beta hydroxy delta 5 steroid dehydrogenase) mRNA expression at normoxia but decreased their expression under hypoxia at 3 nM and 5 nM concentrations (Fig. 3a,b). Interestingly, echinomycin caused a dose-dependent downregulation of cytochrome P450 Family 19 Subfamily A Member 1 (*CYP19A1*; Fig. 3c) expression under both oxygen conditions.

Cell Proliferation. Expression of cyclin D2 (*CCND2*) and *PCNA* were analyzed to determine the effect of HIF1 on cell proliferation. Echinomycin mediated inhibition of HIF1 function resulted in the downregulation of *CCND2* (Fig. 4a) and *PCNA* (Fig. 4b) under both normoxic and hypoxic conditions.

Effects of HIF1A knockdown in granulosa cells. GC health was unaffected by the *HIF1A* gapmer at 50 nM concentration as there were no significant changes in the viability, apoptosis, and dead cell status (Supplementary Information: S5,S6). qPCR quantification showed that *HIF1A* mRNA expression was significantly knocked down under normoxic and hypoxic conditions by the *HIF1A* gapmers (Fig. 5a). Similar to the echinomycin treatment, expression of *VEGFA* was downregulated (Fig. 5b) and *VNN2* was upregulated upon *HIF1A* knockdown (Fig. 5c).

Steroidogenesis. Suppression of *HIF1A* expression caused a consistent downregulation of *STAR* expression both under normoxic and hypoxic conditions (Fig. 6a). A marginal yet significant upregulation of *HSD3B* expression was detected at normoxia (Fig. 6b), but no changes have been observed under hypoxia. *CYP19A1* expression was significantly downregulated at normoxia (Fig. 6c) while no such effects were observed at hypoxia with *HIF1A* knockdown. Estimation of steroids using radioimmunoassay specified a marginal yet significant decrease in the production of estradiol at normoxia and progesterone at hypoxia (Fig. 6d,e) upon knocking down *HIF1A* expression. The effects were not significant concerning estradiol and progesterone production under hypoxic and normoxic conditions, respectively.

Cell proliferation. Similar to the effects of echinomycin, *HIF1A* knockdown induces downregulation of cell proliferation markers *CCND2* and *PCNA* under normoxic conditions (Fig. 7a,b). However, *PCNA* expression was increased upon *HIF1A* knockdown at hypoxia. These effects were further analyzed using flow cytometry, which indicated that knockdown of *HIF1A* leads to decreased and increased numbers of cells in DNA replication stage under normoxic (Fig. 7c) and hypoxic (Fig. 7d) conditions, respectively.

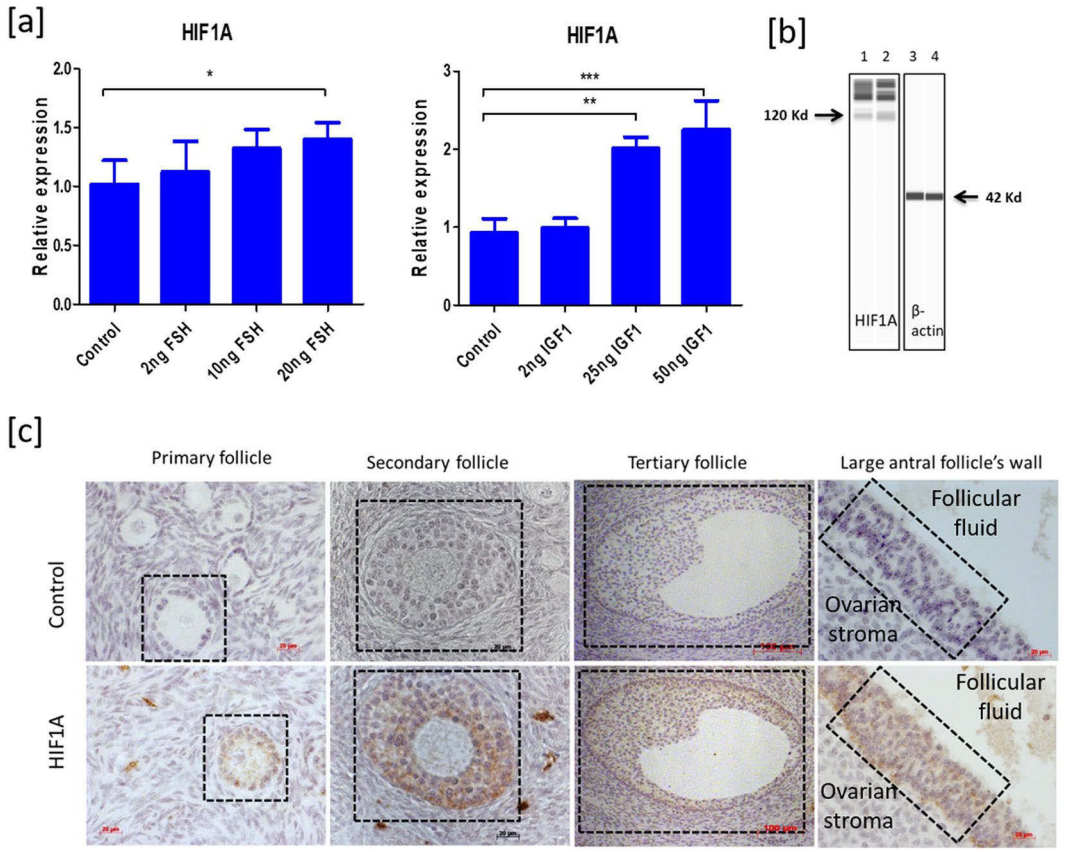

Figure 1. Expression analysis of HIF1A in granulosa cells. (**a**) indicates the mRNA expression of HIF1A under different FSH and IGF1 concentrations. (**b**) Indicates the detection of HIF1A protein under normoxia. The lane numbers 1–4 indicate the western runs of individual protein lysates. Columns 1 and 2 represent duplicates of HIF1A probing in FSH (20 ng/ml) and IGF1(50 ng/ml) treated GC under normoxia while columns 3 and 4 represent the Beta actin (BACT) probing in the corresponding samples. The arrow marks in (**b**) indicate the HIF1A (columns 1 and 2) and BACT (columns 3 and 4) protein bands. The image is obtained with the exposure time of 8 seconds in Simple Western instrument. (**c**) illustrates the immunolocalization of HIF1A in different growing ovarian follicles of abattoir ovaries. The brown color immunochemical signal indicates the HIF1A staining inside the follicle. 400x optical magnification was used for primary and secondary and large antral follicle's wall. 100x optical magnification was used for tertiary follicles in the pictures. Inside dotted box indicates the specific follicle for primary, secondary and tertiary follicle sections whereas dotted box points the GC layer in large antral follicle's wall. Data were presented in Mean ± SEM values of three independent cell culture experiments (n = 3) for Fig. 1a. Significant differences were acknowledged at the minimum level of $p < 0.05$ by one way repeated measures analysis of variance. Pairwise comparisons were analyzed using Post hoc Tuckey test.

Chromatin immunoprecipitation analysis. *CYP19A1* showed a consistent and robust downregulation both in echinomycin and knockdown studies upon affecting HIF1 activity and expression. Sequence analysis of the *CYP19A1* proximal promoter P2 (Fig. 8a) indicates the availability of a putative HIF1 binding site, which is recognized based on the conserved HIF1 binding sequence reported in JASPER database (http://jaspar.genereg.net) (Fig. 8b), similar to human CYP19A1 promoter[24]. Therefore, we analyzed the binding of HIF1A in the bovine *CYP19A1* proximal promoter region to derive possible cues on the regulation of *CYP19A1* expression by HIF1 under FSH and IGF1 stimulated conditions. The results indicate the binding of HIF1 transcription factor in the *CYP19A1* promoter region, which can be seen in the agarose gel images of PCR products (Fig. 8c). Further, substantial binding of anti RNA polymerase antibody to the *CYP19A1* promoter indicates an active transcription of the *CYP19A1* gene in granulosa cells under FSH and IGF1 treated conditions (Fig. 8d,e).

Discussion

The present study is carried out understand the transcriptional activity of HIF1 in GC isolated and cultured from the small antral follicles. It is well known that FSH and IGF1 induced steroidogenesis and proliferation of GC is obligatory for ovarian follicle development in many mammals, including cows[25,26]. Therefore supplementation of FSH and IGF1 is universally practiced for GC cultures, especially for attaining an "estradiol active" phenotype[27–31]. In the present study, treatment of GC with physiologically relevant doses of FSH (20 ng/ml) and IGF1 (25 and 50 ng/ml) independently induced the mRNA expression of *HIF1A*. Therefore, *HIF1A* levels reported

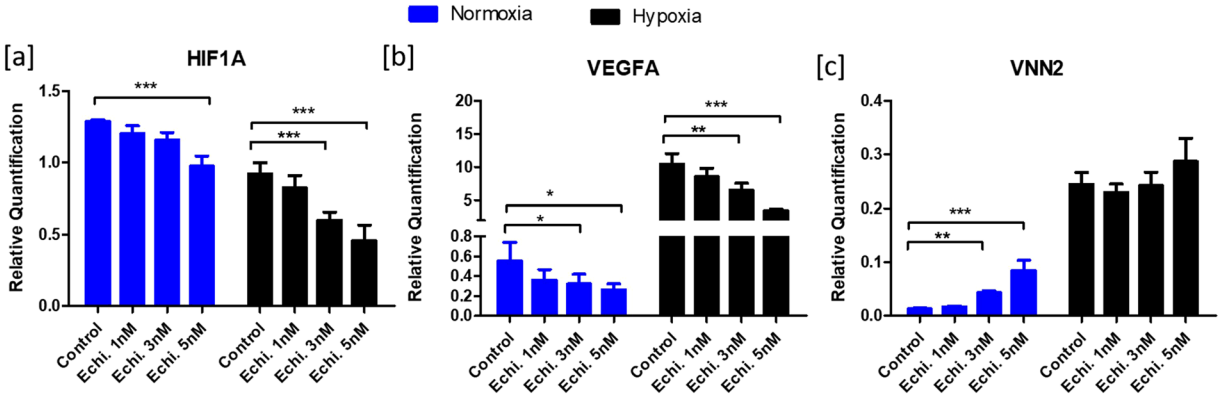

Figure 2. Effects of echinomycin on candidate gene expression. Suppression of HIF1 activity using echinomycin altered the mRNA expression of *HIF1A* (**a**), *VEGFA* (**b**) and *VNN2* (**c**) genes under normoxic and hypoxic conditions. Data were presented in Mean ± SEM values of three independent cell culture experiments (n = 3). Significant differences were acknowledged at the minimum level of $p < 0.05$ by one way repeated measures analysis of variance. Pairwise comparisons were analyzed using Post hoc Tuckey test.

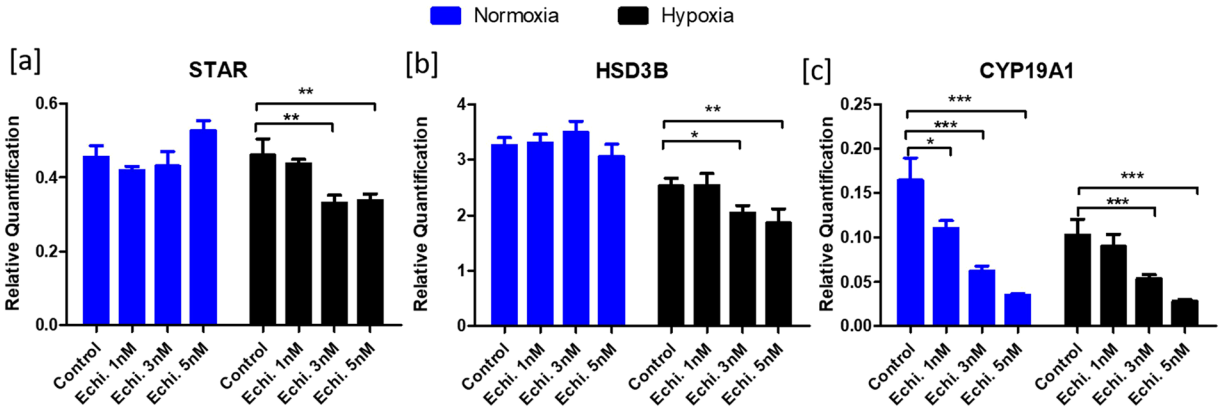

Figure 3. Effects of echinomycin on the expression of steroidogenic genes. Figure depicts the normalized mRNA expression values of *STAR* (**a**), *HSD3B* (**b**) and *CYP19A1* (**c**) genes under normoxic and hypoxic conditions. Data were presented in Mean ± SEM values of three independent cell culture experiments (n = 3). Significant differences were acknowledged at the minimum level of $p < 0.05$ by one way repeated measures analysis of variance. Pairwise comparisons were analyzed using Post hoc Tuckey test.

in developing ovarian follicles[14,15,32] and in the cultured GC[22] could be because of the actions of FSH and IGF1 signaling. However, other endocrine factors such as pregnant mare serum gonadotropins also shown to induce HIF1A expression in ovarian follicle[13], suggesting the physiological state of the animals plays a role in HIF1A expression in ovary.

In the present study, the loss of HIF1 function in GC was studied using echinomycin and *HIF1A* gene knockdown approaches under normoxic and hypoxic conditions. Echinomycin competitively inhibits the transcription factor activity of HIF1 by binding to hypoxia responsive elements (HRE) on the DNA. Surprisingly, mRNA expression of *HIF1A* was downregulated upon echinomycin treatment. This might be because several miRNAs as miR-200, miR-153-3p, miR-429, and miR-18a have been reported to suppress the gene expression of HIF1A in different cells types[33–36]. On the other hand, Shen *et al.* (2013) have indicated that HIF1 stabilization could inhibit miR-200b expression[37]. Therefore, it might be possible that echinomycin mediated suppression of HIF1 activity may enable the expression of miR-200 and of other miRNAs that could target *HIF1A* mRNA. A similar lower expression of *HIF1A* was observed under hypoxic conditions compared to normoxia. HIF1A protein is well known to have less stability under normoxic conditions. Whereas under hypoxic conditions, HIF1A protein is stabilized due to inhibition of prolyl hydroxylase activity[2,38]. Therefore we speculate that the HIF1A mRNA expression under hypoxic conditions might also be subjected to a feedback regulation compared to the normoxic conditions and result in a bit lower expression in hypoxia. In any case, further dedicated experiments are needed to understand the regulation of transcript abundance of *HIF1A* in GC under normoxic and hypoxic conditions and echinomycin treatment.

Loss of HIF1 function consistently resulted in the downregulation of *VEGFA*, which is a well-established downstream effector of *HIF1A* action in different cell types. Most importantly, this particular effect on *VEGFA*

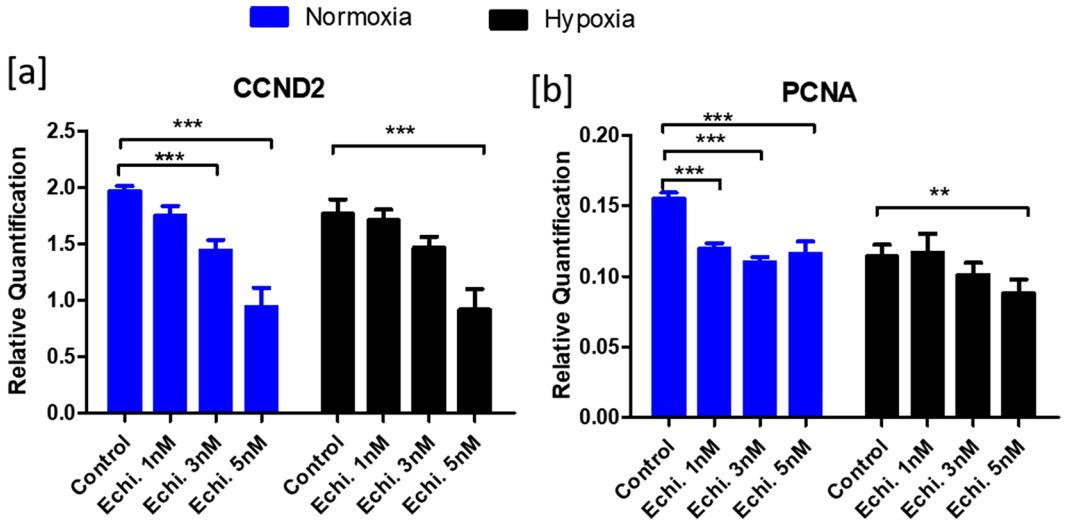

Figure 4. Effects of echinomycin on cell proliferation markers: Figure depicts the normalized mRNA expression data of *CCND2* (**a**) and *PCNA* (**b**) genes upon inhibition of HIF1 activity using echinomycin. Data were presented in Mean ± SEM values of three independent cell culture experiments (n = 3). Significant differences were acknowledged at the minimum level of $p < 0.05$ by one way repeated measures analysis of variance. Pairwise comparisons were analyzed using Post hoc Tuckey test.

expression confirms the suppression of HIF1 activity in GC after the treatment with echinomycin and anti-*HIF1A* gapmer and also indicates the functionality of HIF1 both at normoxic and hypoxic environments. A similar finding regarding FSH induced expression of *VEGFA* in GC is shown to be mediated via HIF1 in mice[39]. In the present study, VEGFA has shown a lower expression in normoxia compared to hypoxia possibly because of the lower stability of HIF1A protein under normoxic conditions compared to hypoxia. However, it is possible that *VEGFA* expression could also depend on other companion factors such as activator protein 1 (AP1) and specificity protein 1 (SP1)[40], whose expression was not analyzed in the present investigation.

VNN2 has been reported to be involved in inflammatory processes by inducing leukocyte migration and adhesion processes in physiological conditions[41]. We have analyzed the expression of *VNN2* as it was shown to be upregulated in GC isolated from pre-ovulatory follicles after LH surge[42], which are implied to be on the edge of severe hypoxic conditions[19]. Our data revealed that the expression of *VNN2* is upregulated by hypoxia in GC. But knockdown of *HIF1A* mRNA abundance downregulated the expression of *VNN2* both under normoxic and hypoxic conditions. This important observation suggests that the upregulation of *VNN2* in GC of the ovulatory follicle maybe because of the prevailing hypoxic conditions rather than actions of LH. Importantly, the inhibitory action of HIF1 on *VNN2* suggests its anti-inflammatory actions under FSH and IGF1 Supplemented conditions[43].

The expression of three key steroidogenic genes (*STAR*, *HSD3B*, and *CYP19A1*) was analyzed upon inhibiting the HIF1 function. *STAR* catalyzes a rate-limiting process of transporting cholesterol from the outer into the inner mitochondrial membrane in steroidogenic cells. *HSD3B* catalyzes the conversion of pregnenolone into progesterone. The *CYP19A1* coded aromatase converts androgens into estrogens, which are essential female sex hormones[44]. Regulation of these three steroidogenic genes by *HIF1A* appears to be dynamic and tissue-dependent as revealed by different studies. The expression of STAR was shown to be induced by HIF1 in mouse KK1 cells[18]. Conversely, the inhibitory action of HIF1 on *STAR* expression is documented in mouse Leydig cells[45]. Similar reports can be found that HIF1 could increase *HSD3B* expression in Leydig cells[46], while others suggest that it may inhibit *HSD3B* and progesterone production in canine luteal cells[47]. Likewise, HIF1 has been shown to induce *CYP19A1* expression in breast adipose stromal cells[24] and inhibits the same in H295R cells, which are steroidogenic adrenal cortical cells[48]. In the present study, we could show that inhibition of HIF1 function leads to dose-dependent suppression of *CYP19A1* expression and estradiol production in bovine GC under normoxic conditions. Indeed, a chromatin precipitation analysis indicated significant binding of HIF1 to the *CYP19A1* proximal promoter in breast adipose stromal cells[24]. Our results are in agreement with this earlier study as we could see the binding of *HIF1* on *CYP19A1* proximal promoter in chromatin precipitation analysis. Therefore, the present results suggest that *CYP19A*1 is a plausible HIF1 target gene in granulosa cells of developing follicles, especially under FSH and IGF1 signaling conditions. However, this stimulatory effect of HIF1 on *CYP19A1* expression and estradiol production was not seen under hypoxic conditions upon *HIF1A* knockdown possibly because of the attenuation of FSH and IGF1 signaling. HIF1 has been shown to be involved in the hypoxic synthesis of progesterone in GC[49]. Our results are in the scope of this finding as inhibition of HIF1 activity decreased the progesterone production at hypoxia by possibly inhibiting *STAR* and *HSD3B* expression. We observed an apparent discrepancy between echinomycin and gapmer treatments concerning *STAR* and *HSD3B* gene expression. Echinomycin did not alter STAR expression under normoxia but could significantly decrease the same under hypoxia. In contrast, *HIF1A* knockdown consistently decreased *STAR* expression both under normoxic and hypoxic conditions similar to murine GC[18].

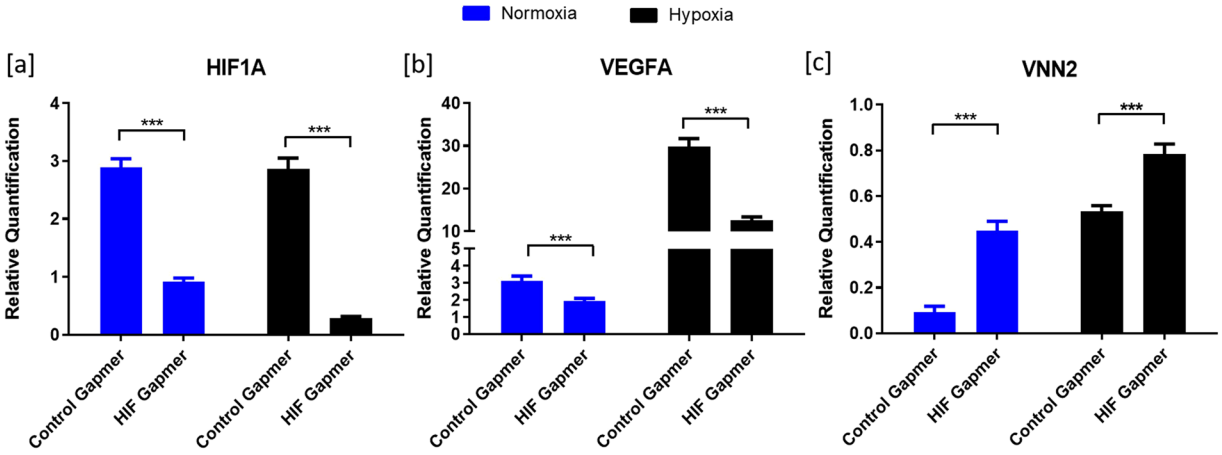

Figure 5. Effects of HIF1A knockdown on candidate gene expression. Figure depicts the mRNA expression values of *HIF1A* (**a**), *VEGFA* (**b**) and *VNN2* (**c**) genes upon *HIF1A* knockdown under normoxic and hypoxic conditions. Data were presented in Mean ± SEM values of three independent cell culture experiments (n = 3). Significant differences were acknowledged at the minimum level of $p < 0.05$ by t-test.

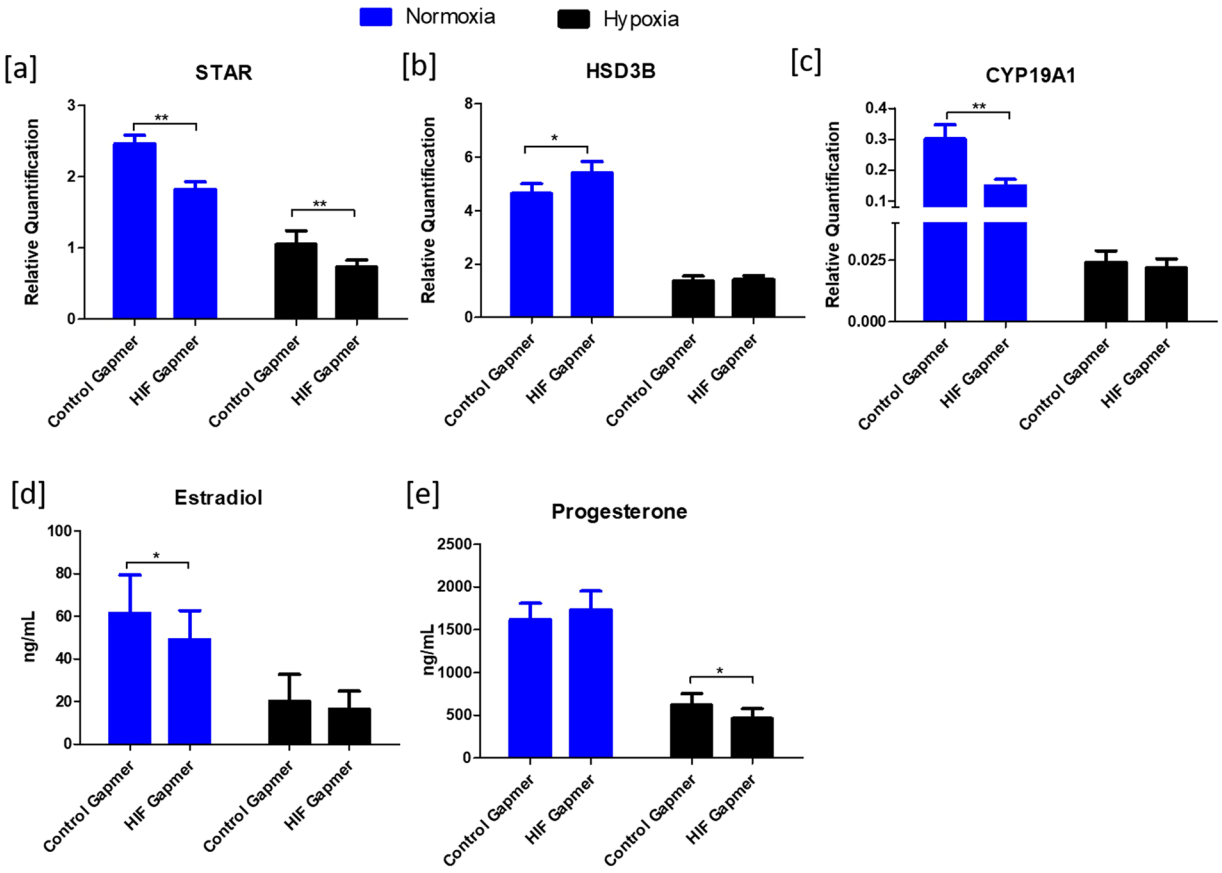

Figure 6. Effects of HIF1A knockdown on the expression of steroidogenic genes and steroidogenesis. Suppression of HIF1 expression by knockdown altered *STAR* (**a**), *HSD3B* (**b**) and *CYP19A1* (**c**) transcript abundance. (**d**,**e**) show the estradiol and progesterone concentrations in the spent culture media. Data were presented in Mean ± SEM values of three independent cell culture experiments (n = 3). Significant differences were acknowledged at the minimum level of $p < 0.05$ by t-test.

PCNA and *CCND2* are well known positive markers of cell proliferation in many cells. In the ovary, both genes might play an essential role in follicular maturation as the proliferation of GC is an essential feature of follicular growth and development[13]. Moreover, *CCND2* and *PCNA* have been used in a number of studies to mark the

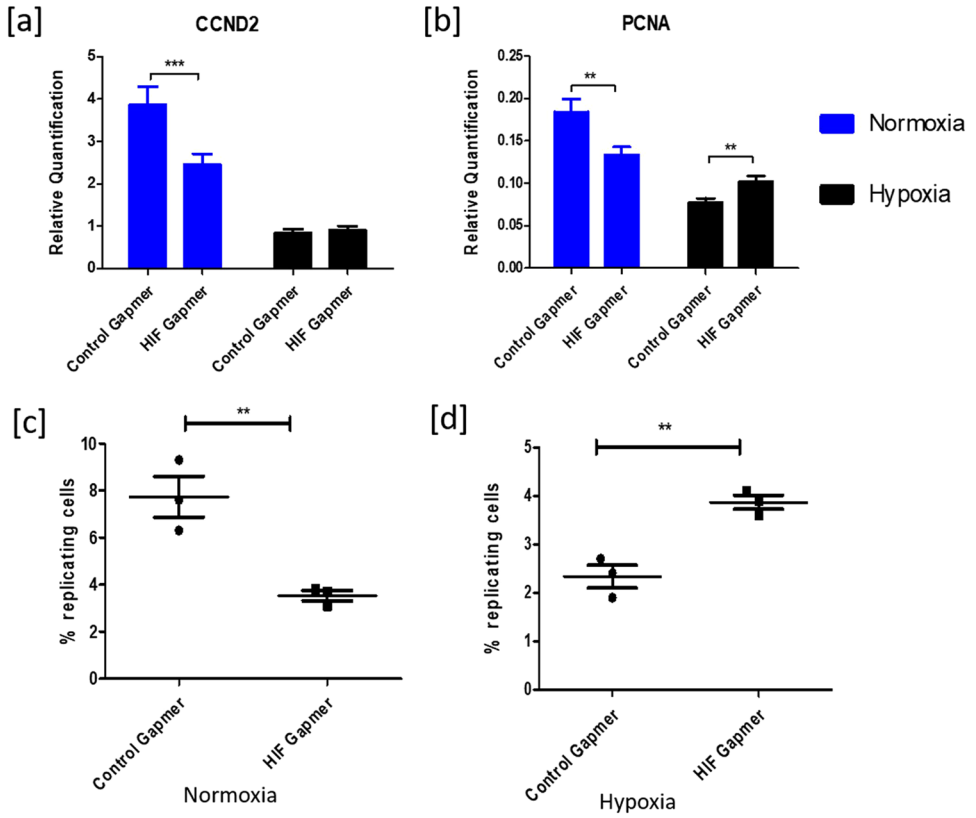

Figure 7. Effects of HIF1A knockdown on cell proliferation. (**a**,**b**) depicts CCND2 and PCNA mRNA expressions upon HIF1A knockdown in granulosa cells under normoxic and hypoxic conditions. (**c**,**d**) depict the percent of replicating cells (S-phase) under normoxic and hypoxic conditions, respectively, upon treating with control and HIF1A gapmers. Data were represented in the mean ± SEM values of three independent culture experiments (n = 3). Significant differences were acknowledged at the minimum level of $p < 0.05$ by t-test.

proliferation status of GC *in-vitro* and *in-vivo*[42,50,51]. Expression of *PCNA* and *CCND2* was downregulated at normoxia and hypoxia in the presence of either echinomycin or *HIF1A* gapmers, indicating the contribution of HIF1 in GC proliferation under normoxic conditions. However, the effects of echinomycin on these genes appeared to be more prominent at all tested concentrations in normoxia than hypoxia likely because of the higher proliferation of GC under normal oxygen levels[22]. Most importantly, these results indicate the active role of HIF1 in FSH and IGF1 induced proliferation of GC. A similar downregulation of *PCNA* expression upon HIF1 inhibition has been documented in rat GC under normoxic conditions[13]. On the other hand, it has been shown that HIF1 induces cell cycle arrest at hypoxia in different cell types[52,53]. Similarly, we could observe an upregulation of *PCNA* gene expression at hypoxia following knockdown of *HIF1A* expression. However, similar to *STAR* and *HSD3B* genes we did not see this similar regulation in echinomycin treatment. In any case, it might be important to acknowledge the plausible off-target effects induced by chemical inhibitors under different circumstances[54]. Fluorescence-activated cell sorting (FACS) analysis has further confirmed that HIF1 knockdown could induce cell cycle progression under hypoxia. Based on these data, we could interpret that HIF1 conversely regulates cellular proliferation between normoxic and hypoxic conditions. To the best of our knowledge, this is the first report to show differential regulation of cell proliferation by HIF1 under normoxic and hypoxic conditions. We further speculate that the function of HIF1 might be dependent upon the original stimulus that induces HIF1 function. For instance, FSH and IGF1 induced HIF1 function at normoxia is associated with GC proliferation together with other companion signaling molecules. If the stimulus is hypoxia, HIF1 might be involved in the suppression of cell proliferation[55] as cells do not have an adequate supply of energy resources such as ATP under hypoxic conditions. These novel insights into HIF1 function could be valuable to understand that differential modulation of the activities of effector molecules depends on the initial stimulus. However, future omics based investigations are needed to further understand the differential role of HIF1 under both normoxic and hypoxic conditions.

Overall, the present study provides a molecular cue regarding the transcriptional regulation of HIF1 in GC both under normoxic and hypoxic conditions. We want to acknowledge that these results may represent the regulation by HIF1 in developing follicle where the GC show an estrogen active phenotype. We also acknowledge that the results under hypoxia are not representative of the situation in matured preovulatory follicles as we did not treat the cells with LH. However the data provide interesting and novel clues regarding the regulation of genes in GC by HIF1 under hypoxia.

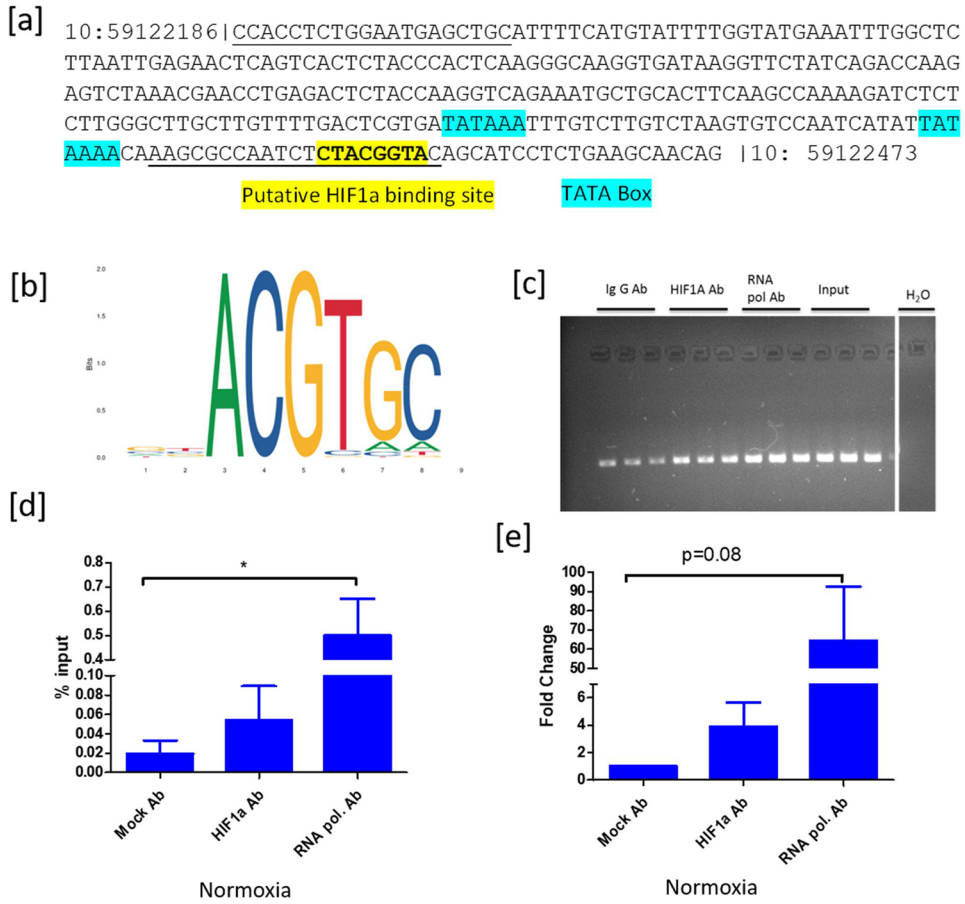

Figure 8. Chromatin immunoprecipitation analysis. (**a**) shows the nucleotide sequence between the physical location of 59122186 to 59122473 nucleotides on chromosome 10 in the bovine genome. Manually curated TATA box and the putative HIF1 binding sites were highlighted in blue and yellow colors. The primer binding sites were indicated with an underline. (**b**) adopted JASPAR Sequence logo of the HIF1 binding site based on ChIP seq data in humans. (**c**) indicates the agarose gel pictures of immune-precipitated and input DNA samples. (**d**,**e**) depict the relative quantification of HIF1 binding events on the CYP19A1 promoter based on percent input and fold change methods, respectively under FSH and IGF1 Supplemented conditions. Data were represented in mean ± SEM values of three independent experiments (n = 3). Significant differences were acknowledged at the minimum level of $p < 0.05$ by one way repeated measures analysis of variance. Pairwise comparisons were analyzed using Post hoc Tuckey test.

Conclusions

Our results indicate that HIF1 plays an essential role in bovine follicular development as HIF1 driven transcriptional activity could play a modulatory role in steroidogenesis and cell proliferation of cultured bovine GC, whereby HIF1 seems to act differentially on some target genes under normoxic vs. hypoxic conditions. Estradiol and progesterone production may be differentially regulated by HIF1 under normoxic and hypoxic conditions, possibly due to the inhibition of FSH signaling under hypoxia. Overall, our data suggest that HIF1 driven transcriptional activity plays a crucial role in GC functionality in bovine ovarian follicles (Fig. 9).

Materials and Methods

Cell culture. Bovine ovarian samples used for obtaining the GC are collected from a local abattoir and does not require ethical permission according to the German law. The number of ovaries for each lot of collection (usually 8 to 12 ovaries) depends upon the availability of animals at the abattoir. Therefore the number of animals and ovaries used for the experiments is varied in general but assures the heterogeneous population of primary GC in each cell culture experiment. Ovaries were collected in ice-cold 1x PBS containing antimicrobials such as streptomycin (0.1 mg/ml), penicillin (100IU) and amphotericin (0.5 µg/µl). GC were aspirated from the ovaries with a syringe and 18 gauge needle and cryopreserved in freezing medium consisting of 90% fetal calf serum and 10% Dimethyl sulfoxide. For cell culture experiments, cryopreserved cells were thawed in a water bath, washed and re-suspended in α-MEM, and plated at a density of ~1.4×10^5 viable cells, according to the trypan blue exclusion assay, in each well of 24 well culture dishes, which were pre-coated with 0.02% collagen R (Serva, Heidelberg). The α-MEM was reconstituted with 0.084% sodium bicarbonate, 0.1% BSA, 20 mM HEPES, 2 mM L-Glutamin, 4 ng/ml sodium selenite, 5 µg/ml transferrin, 10 ng/ml insulin, 1 mM non-essential amino acids, 100 IU penicillin

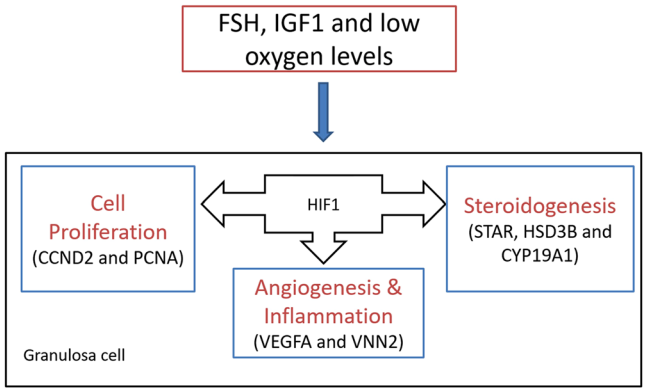

Figure 9. HIF1 is essential for granulosa cell function. Expression of HIF1A is induced by FSH, IGF1 and low oxygen levels in bovine granulosa cells. HIF1 may regulate steroidogenesis, cell proliferation, angiogenesis and inflammation processes in developing ovarian follicles.

and 0.1 mg/ml streptomycin. Additionally, 50 ng/ml IGFI (Sigma Aldrich, Steinheim, Germany), 20 ng/ml FSH (Folltropin-V, Vetoquinol; Sigma Aldrich, Steinheim, Germany), and 2 μM androstenedione (Sigma Aldrich, Steinheim, Germany) were added to the α-MEM before plating the cells. For identifying the effect of FSH and IGF1 on *HIF1A* mRNA expression under normoxia, culture media were prepared with either FSH (20 ng/ ml) or IGF1 (50 ng/ ml). For normoxic conditions, culture plates were maintained in a CO_2 incubator at 21% O_2 and 5% CO_2 for 8 days while spent media was replaced every 2nd day. For hypoxic conditions, a subset of culture plates was incubated at 1% O_2 and 5% CO_2 on day 6 for 48 hrs. α-MEM was supplemented with different concentrations of (1 nM, 3 nM and 5 nM) echinomycin on day 6 to inhibit *HIF1*. For *HIF1A* knockdown, GCs were transfected with 50 nM of antisense LNA negative control gapmer (A*A*C*A*C*-G*T*C*T*A*T*A*C* G*C), and anti *HIF1A* gapmer (A*C*T*G*A*T*C*G*A*A*G*G*A*-A*C*G) using TransIT-X2® transfection reagent (Mirus Bio, USA) as per the manufacturer's recommendations in a 24 well culture dishes for 6 days (from day 2 to day 8 of the culture). As established before, all cultured cells were subjected to different analyses on day 8[20–23].

RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis. Total RNA was isolated using innuPREP RNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's recommendations. The eluted RNA was quantified using a NanoDrop1000 Spectrophotometer (Thermo Scientific, Bonn, Germany). 200 ng total RNA was used for first-strand cDNA synthesis using SensiFAST cDNA Synthesis Kit (Bioline, Luckenwalde, Germany). Quantitative real-time PCR (qPCR) was performed using a light cycler 96 instrument (Roche, Mannheim, Germany). All PCR products amplified with the different primers (Supplementary Information: S1) were initially cloned in a pGEM-T vector (Promega biosciences, USA) and sequenced to verify primer pair specificity. If the product is correct, cloned vectors at five different serially diluted concentrations (5×10^{-12} to 5×10^{-16} g plasmid) were used for generating the standard curve in each run of qPCR. The abundance of transcripts was normalized using mean expression values of RPLP0 and TBP housekeeping transcripts. The melting curve analysis followed by agarose gel (3%) electrophoresis of PCR products was performed to ensure the correct identity of amplicons (Supplementary Information: S2).

Capillary western blot. Western blots were executed using a WES instrument (Protein simple, CA, USA) according to the manufacturer's guidelines. Briefly, cultured cells were lysed on ice using 1x RIPA buffer (Thermo Fischer, USA) followed by sonication and centrifugation steps to collect the protein supernatant. Protein concentrations in the supernatant were measured using BCA protein estimation assay kit (Thermo Fischer, USA). Protein samples (2 μg), wash buffers, blocking reagent, antibodies, and chemiluminescent substrate were prepared and distributed into the appropriate wells of assay plates. Subsequently, assay plates were loaded to the WES instrument, and proteins were allowed to separate. Detection of bands was performed automatically in the individual capillaries by the WES instrument. Anti HIF1A primary antibody (Novus biologicals, USA; Catalog# NBP1-02160SS; 1:250) and anti-beta actin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Catalog No sc-47778: 1:250) were used together with anti-rabbit and anti-mouse secondary antibody supplied by ProteinSimple.

Immunolocalization of HIF1α in ovarian follicles. Four bovine ovaries were obtained from a local abattoir and sliced into small pieces for fixation in Bouin's reagent (10 ml of 37% formalin, 50 ml of glacial acetic acid and 150 ml of picric acid) for two days. Tissue samples were then dehydrated in a gradient series of ethanol and subjected to paraffin embedding using an MPS/W instrument (SLEE medical GmbH, Germany). 5 μm sections were prepared using a microtome and mounted into glass slides. Sections were deparaffinized and blocked with 2% BSA for 1 hr and incubated with either anti-HIF1A antibody (Invitrogen, Catalog No. MA1-16504; 1:100) in 1% BSA or 1% BSA (without antibody) as control overnight. Subsequently, slides were washed four times with wash buffer and incubated with secondary rabbit anti-mouse IgG(F(ab′)2) antibody (Sigma Aldrich, catalog No. AQ160B) for 1 hr. Slides were then treated with streptavidin HRP conjugate (Sigma, Catalog No. 1089153) for 40 min followed by incubation with HRP substrate, DAB, for 30 sec. All slides were counterstained with mayers

hemalum solution for 10 sec. Finally, Roti mount aqua solution was added on each slide for color protection, and images were obtained using a bright-field with Axio imager A1 microscope (Carl Zeiss Inc, Germany).

Cell viability assay. The spent media were collected in 1.5 ml tubes and centrifuged to pellet the floating dead cells. Adherent GC were washed twice with 1x PBS and detached using 250 µl of tryplE reagent per each well. The detached cells were merged with floating cells to ensure the inclusion of dead cell portions. Cells were washed with 1 ml α-MEM and resuspended in 100 µl of 1x binding buffer. 10 µl of Annexin V reagent was added to the cell suspension and incubated in the dark for 15 min followed by washing to remove the unbound dye and resuspension in 500 µl of 1x binding buffer. 5 µl of propidium iodide (500 µg/ml) was added to the cells just before flow cytometric analysis. The fluorescence signals from single cells were quantified by using a flow cytometer (Gallios, Beckman-Coulter) and the data were analyzed using the Kaluza software (Beckman-Coulter).

Steroid hormone estimation. The concentrations of estradiol and progesterone in the spent media were measured through a competitive 3H–radioimmunoassay (RIA) using custom generated purified rabbit-raised antibodies. [2, 4, 6, 7-3 H] 17β-estradiol (GE Healthcare, Freiburg, Germany) and [1, 2, 6, 7-3 H (N)] progesterone (PerkinElmer, Boston, USA) were used as tracer molecules for E2 and P4 estimation, respectively. The minimum detection limit for E2 and P4 was 3 pg/ml and 7 pg/ml respectively with inter and intra assay coefficient of variation of 9.9% and 6.9% for estradiol, and 7.6% and 9.8% for progesterone. The standards were prepared by dissolving E2 and P4 in 100% ethanol which was subsequently diluted in RIA buffer. Spent media were diluted 1:40 in RIA buffer for P4 estimation and used undiluted for E2 estimation. All measurements were executed in duplicates. The radioactivity levels were analyzed in a liquid scintillation counter (TriCarb 2900 TR; PerkinElmer) with an integrated RIA-calculation program.

Cell cycle assay. The number of cells in the proliferation was determined based on the detected DNA fluorescence in flow cytometric analysis. Cells were detached by adding 250 µl TryplE reagent (Thermo Fischer, USA) to each culture well. Subsequently, cells were washed and resuspended in 300 µl of 1x PBS. The cell suspension was dropwise added into 70% ice-cold ethanol and stored at −20 °C for 2 hr. Later, cells were pelleted at 300 g for 10 min and incubated in 1 ml of RNase solution (1 mg/ml) at 37 °C for 30 min. Propidium iodide reagent (50 µg/ml) was added to the cells and incubated in the dark at 37 °C for 30 min. The fluorescence signal was quantified from single cells (10,000 counts) using an EPICS-XL flow cytometer (Beckman-Coulter, Krefeld, Germany). Data were analyzed using the Multicycle software (Phoenix, USA).

Chromatin immuno-precipitation (ChIP). ChIP was performed using the MAGNA ChIP kit (Millipore) by following the manufacturer's instructions. The cells were cultured in 60 cm^2 culture plates at an initial seeding density of 40×10^5 cells/plate in 12 ml of α-MEM, supplemented with 50 ng/ml IGF1 and 20 ng/ml FSH for 8 days while α-MEM was replaced every 2nd day. On day 8, 1% formaldehyde was added to the cultured plates to crosslink the chromatin with proteins. After 10 min, cells were treated with 10x glycine for quenching the unreacted formaldehyde in the media. The cells were then washed twice with 1x PBS and collected into a 1.5 ml collecting tube using a cell scraper. The cells were then pelleted and dissolved in lysis buffer containing protease inhibitor cocktail (PIC). The cells in lysis buffer were sonicated using a Covaris S220 instrument according to the manufacturer's recommendation. For immunoprecipitation, 450 µl of dilution buffer containing PIC was added to 50 µl of sonicated DNA. From this, 5 µl was aliquoted for input DNA analysis and stored at 4 °C. 5 µg of anti-HIF1A antibody (Invitrogen, Catalog #. MA1-16504) and 20 µl of fully suspended protein AG beads were added to the remaining solution followed by incubation overnight on a rotator at 4 °C. Unspecific mouse Ig G (Merck Millipore) and specific anti-RNA polymerase antibody (Merck Millipore) were used as a negative and positive control, respectively. On the next day, chromatin-antibody-magnetic bead complexes were washed with a series of wash buffers such as low salt, high salt, lithium chloride and Tris EDTA buffer, provided in the Magna ChIP kit. All protein-DNA complexes and inputs were subjected to reverse cross-linking by adding elution buffer containing proteinase K and incubated for 2 hr at 62 °C. DNA was eluted using silica-based polypropylene spin columns supplied in the MAGNA kit. The anti *HIF1A* antibody precipitated DNA was subjected to amplification of the proximal promoter (P2) region of *CYP19A1*[56,57] together with positive and negative controls. SYBR green chemistry was used for the quantification of HIF1 binding. Percent input and ddCt methods were used to quantify the relative amount of HIF1A binding events on *CYP19A1* promotor.

Statistical analysis. Analyses in the present study were executed from different cell preparations with three independent primary granulosa culture experiments. Untransformed data values have been used for data analysis. Statistical interpretations were derived using Graphpad Prism software. Data were analyzed using one-way repeated measures analysis of variance. Pairwise multiple comparisons were executed using post hoc Tukey's test. Student t-test was applied for the comparisons between two groups (e.g., control gapmer Vs. HIF1A gapmer). Data is presented as MEAN ± SEM values in all figures and tables. Probability (p) values < 0.05 were considered as statistically significant and are designated with up to three asterisk symbols to inform the strength of significant difference (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$).

Data availability

All the data and materials of the present study have been presented in the manuscript and in the Supplementary Information.

Received: 28 November 2019; Accepted: 19 February 2020;
Published online: 03 March 2020

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Acknowledgements

Authors acknowledge Maren Anders, Veronika Schreiter, Christian Plinski, Petra Reckling and Swanhild Rodewald for their technical support. The study was financially supported by FBN core fund and Alexander von Humboldt Foundation, Bonn, Germany.

Author contributions

V.S.B. and J.V. designed all experiments. V.S.B., A.S. and M.M. executed experiments. V.S.B. and J.V. wrote the manuscript. All authors have given a significant contribution to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-60935-1.

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4. Summary

The present dissertation investigated effects imposed by high NEFA levels on cultured follicular GCs. SFAs and UFAs elicited differential, partly opposing changes in the expression of genes involved in steroidogenesis, cell proliferation, viability and cell identity. PA and SA had stimulatory effects on steroidogenesis, while OA, ALA and *cis-9, trans-11* CLA had inhibitory effects on the same. However, in a mixed pool of fatty acids the effects of OA dominated with reduced E2 production. Furthermore, the effect of OA on GC function was validated *in vivo* in heifers by directly injecting OA into dominant follicles. As observed *in vitro*, the E2 concentration was reduced in the FF recovered post OA injection and also the ovulation tended to be inhibited. Dietary supplementation of EFAs to lactating German Holsteins lead to a substantial increase of ALA and *cis-9, trans-11* CLA in the FF, however without significantly affecting the *in vitro* oocyte competence. In the GC culture model however, inhibitory effects of ALA and *cis-9, trans-11* CLA were observed on steroidogenesis and viability. Another serious metabolic challenge for the inner non-vascularized GC layer of growing follicles is the increasing O_2 deficiency with growing diameter. Experiments in cultured GCs revealed that luteinization events such as down-regulation of FSH signaling, of cell proliferation and of steroidogenesis, and up-regulation of genes involved in angiogenesis, glucose metabolism and inflammatory processes are promoted when these cells are exposed to hypoxia. This suggests that hypoxia in preovulatory follicle contributes to early events of luteinization. In addition it could be demonstrated that under hypoxic and normoxic conditions the HIF1 transcription factor might be involved in regulating cell proliferation and steroidogenesis in bovine GCs as indicated by suppression of HIF1A using echinomycin or gapmer gene knockdown approaches.

To sum up, physiological stressors can remarkably affect ovarian function by either promoting or impeding certain metabolic processes. In postpartum dairy cows experiencing metabolic stress in form of higher levels of NEFA in the FF, ovarian GC function can be severely affected, thus leading to delayed estrus resumption or reduced oocyte quality. These findings might be applicable to humans as well, as female patients suffering from obesity, diabetes or PCOS have increased serum concentration of NEFAs, which increases their risk of fertility impairment.

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6. Annexure

1. List of Primers used for transcript quantification by RT-qPCR

Gene	Sequence	Size(bp)	Accession No.
RPLPO	For:TGGTTACCCAACCGTCGCATCTGTA Rev: CACAAAGGCAGATGGATCAGCCAAG	142	NM_001012682
STAR	For: TTGTGAGCGTACGCTGTACCAAG Rev: CTGCGAGAGGACCTGGTTGATG	237	NM_174189.2
CYP19A1	For: GCTTTTGGAAGTGCTGAACCCAAGG Rev: GGGCCCAATTCCCAGAAAGTAGCTG	172	NM_174305
HSD3B1	For: TGTTGGTGGAGGAGAAGGATCTG Rev: GCATTCCTGACGTCAATGACAGAG	208	NM_174343
FSHR	For: TCACCAAGCTTCGAGTCATCCCAAA Rev: TCTGGAAGGCATCAGGGTCGATGTA	189	NM_174061
LHCGR	For:GCATCCACAAGCTTCCAGATGTTACGA Rev: GGGAAATCAGCGTTGTCCCATTGA	205	NM_174381
CD36	For: GCTCCTTAAGCCATTCTTGGAT Rev: CACCAGTGTCAACGCACTTT	151	NM_001278621.1
CCND2	For: CGCAGGGCCGTGCCGGACGCCAAC Rev: CACGGCCCCCAGCAGCTGCAGATGG	279	NM_001076372
PCNA	For: GTGAACCTGCAGAGCATGGACTCGT Rev: CGTGTCCGCGTTATCTTCAGCTCTT	192	NM_001034494
FOXL2	For: AGCCAAGTTCCCGTTCTACG Rev: GGTCCAGCGTCCAGTAGTTG	140	NM_001031750.1
SOX 9	For: ACCTGGAACTTCAGTGGCG Rev: CCAAGTAGGGGAAGGCGAAT	147	XM_010816647.1

2. Proportional fatty acid profiles of follicular fluid samples obtained 48h post intrafollicular injection of BSA vehicle control (BSA) and BSA conjugated oleic acid (OA).

Fatty acid profile by gas chromatography analysis		Follicular fluid proportions (%)		
		BSA	OA	p-value
Saturated fatty acids (SFAs)				
C10:0	(Capric acid)	0.10±0.07	0.09±0.03	0.926
C11:0	(Undecyclic acid)	0.03±0.007	0.02±0.005	0.619
C12:0	(Lauric acid)	0.43±0.07	0.43±0.05	0.950
C13:0	(Tridecyclic acid)	0.13±0.02	0.15±0.02	0.539
C14:0	(Myristic acid)	2.34±0.18	2.41±0.44	0.885
C15:0	(Pentadecyclic acid)	1.76±0.35	1.53±0.14	0.598
C16:0	(Palmitic acid)	19.05±2.00	15.52±2.2	0.304
C17:0	(Margaric acid)	1.75±0.38	1.14±0.20	0.239
C18:0	(Stearic acid)	13.91±0.73	8.06±0.61	0.004
C20:0	(Arachidic acid)	0.59±0.12	0.49±0.08	0.525
C22:0	(Behenic acid)	1.53±0.07	1.44±0.05	0.388
C23:0	(Tricosylic acid)	0.61±0.05	0.36±0.17	0.238
C24:0	(Lignoceric acid)	2.49±0.12	1.92±0.27	0.135
Total SFAs		44.71±3.6	33.56±2.89	0.075
Monounsaturated fatty acids (MUFAs)				
C16:1cis-9	(Palmitoleic acid)	3.56±0.46	1.68±0.54	0.058
C18:1trans-9	(Vaccenic acid)	0.14±0.05	0.14±0.05	0.946
C18:1trans-11	(Elaidic acid)	0.35±0.07	0.12±0.02	0.047
C18:1cis-9	**(Oleic acid)**	**13.59±2.5**	**46.51±5.8**	**0.007**
C18:1cis-11	(Gondoic acid)	0.92±0.37	0.58±0.32	0.934
Total MUFAs		18.55±2.69	49.02±5.08	0.006
Polyunsaturated fatty acids (PUFAs)				
C18:2n-6	(Linoleic acid)	18.61±1.44	8.64±3.33	0.052
C18:3n-6	(Gamma-linolenic acid)	0.33±0.05	0.20±0.10	0.323
C18:3n-3	(Alpha-linolenic acid)	8.84±1.05	3.67±1.58	0.053
C20:3n-6	(Dihomo-gamma-linolenic acid)	2.23±0.27	0.75±0.30	0.024
C20:4n-6	(Arachidonic acid)	3.00±0.34	1.60±0.59	0.111
C20:5n-3	(Eicosapentaenoic acid)	1.53±0.37	0.68±0.40	0.199
C22:5n-3	(Dicosapantaenoic acid)	1.83±0.25	1.78±0.04	0.865
C22:6n-3	(Docosahexaenoic acid)	0.36±0.11	0.10±0.05	0.107
Total PUFAs		36.74±3.32	17.42±6.32	0.054
Total *ω-3* PUFAs		12.57±1.74	6.23±1.99	0.075
Total *ω-6* PUFAs		24.17±1.71	11.18±4.32	0.049

Proportional fatty acid profiles of follicular fluid samples obtained 48h post intrafollicular injection of BSA vehicle control (BSA) and BSA conjugated oleic acid (OA). Fatty acids were determined by gas chromatography. The data are shown as means±SEM, n=3. P-values are from unpaired *t*-test.

3. Ingredients and chemical composition of lactation and dry-off diet

	Diet	
Item (g/kg of DM)	**Lactation**	**Dry-off[1]**
Ingredients		
Corn silage	457	421
Straw	97	223
Compound feed DEFA[2] (granulated)	446	-
Dried sugar beet pulp	-	163
Extracted soy bean meal	-	99
Grain of rye	-	75
Minerals[3]	-	10
Urea[4]	-	9
Chemical composition[5]		
NEL (MJ/kg DM)	7.1	6.5
Crude fat	23	21
Crude fiber	173	219
Crude protein	146	141
Utilizable protein	143	141
NDF	346	423
ADF	197	249

[1]The Dry period diet was fed from wk 6 to 0 before calving

[2]Ceravis AG, Malchin, Germany: 46.5% dried sugar beet pulp, 25.3% extracted soy bean meal, 23.8% grain of rye, 1.4% urea, 1.1% premix cow, 1.00% calcium, 0.37% phosphorus, 0.42% sodium, vitamins A, D3, E, copper, ferric, zinc, manganese, cobalt, iodine, selenium; 9.3% crude fiber, 8.2% crude ash, 1.8% crude fat, 24.1% crude protein, 21.6% NDF, 12.4% ADF, 7.9 MJ NE_L/kg DM

[3]KULMIN®MFV Plus (Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany): 8.5% magnesium, 7.5% phosphorus, 6.5% sodium, 3.5% HCl-insoluble ash, 1.5% calcium, additives: vitamins A, D_3, E, B_1, B_2, B_6, B_5, B_3, B_{12}, B_9, H, zinc, manganese, copper, cobalt, iodine, selenium, saccharomyces cerevisiae

[4]Piarumin® (SKW Stickstoffwerke Piesteritz GmbH, Lutherstadt Wittenberg, Germany): 99 % urea, 46.5 % total nitrogen

[5]German Society of Nutrition Physiology (2001)

4. Fatty acid composition of the supplemented lipids

Fatty acid (%)	Coconut	Linseed	Safflower	Lutalin®
SFA	89.4	10.4	12.3	11.1
6:0	0.93	–	–	–
8:0	9.85	–	–	–
10:0	6.10	–	–	–
12:0	45.5	–	–	–
14:0	16.9	0.02	0.09	–
16:0	6.87	5.78	7.63	6.23
17:0	–	0.03	–	–
18:0	3.10	4.11	3.71	4.19
20:0	0.11	0.19	0.42	0.22
22:0	–	0.13	0.25	0.37
24:0	–	0.09	0.16	0.10
MUFA	8.35	21.5	23.5	29.5
16:1, *cis*-9	–	0.07	0.12	0.09
18:1, *cis*-9	8.35	20.7	22.4	28.3
18:1, *cis*-11	–	0.61	0.66	0.67
20:1, *cis*-11	–	0.16	0.23	0.51
24:1, *cis*-15	–	–	0.11	–
PUFA	1.83	67.1	62.5	3.75
18:2, *cis*-9, *cis*-12	1.83	15.9	62.0	3.52
18:2, *cis*-9, *trans*-12	–	–	–	0.08
18:2, *trans*-9, *cis*-12	–	–	–	0.07
18:3, *cis*-6, *cis*-9, *cis*-12	–	–	0.05	0.07
18:3, *cis*-9, *cis*-12, *cis*-15	–	51.1	0.24	–
20:2, *cis*-11, *cis*-14	–	0.06	–	–
20:3, *cis*-11, *cis*-14, *cis*-17	–	0.02	–	–
22:5, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19	–	–	0.28	–
CLA	< 0.05	0.02	0.32	54.3
18:2, *cis9, trans11* CLA	–	–	0.12	27.2
18:2, *trans10, cis12* CLA	–	0.02	0.20	27.0
18:2, *cis9, cis11* CLA	–	–	–	0.23

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; CLA: conjugated linoleic acid Control (CTRL, n=9): 76 g/d coconut oil (Bio-Kokosöl #665, Kräuterhaus Sanct Bernhard KG, Bad Ditzenbach, Germany) and 0.06 g/d Vitamin E (Covitol®1360, BASF SE, Ludwigshafen, Germany), 1.48 MJ NE_L/d

Essential fatty acids (EFA, n = 9): 78 g/d linseed (DERBY® Leinöl #4026921003087, DERBY Spezialfutter GmbH, Münster, Germany) and 4 g/d safflower oil (GEFRO Distelöl, GEFRO Reformversand Frommlet KG, Memmingen, Germany), comprised 0.06 g/d Vitamin E, 1.57 MJ NE_L/d

Conjugated linoleic acid (CLA, n = 10): 38g/d Lutalin® (BASF SE, Ludwigshafen, Germany) and 0.06 g/d Vitamin E (Covitol®1360, BASF SE, Ludwigshafen, Germany), 0.69 MJ NE_L/d

Essential fatty acids and conjugated linoleic acid (EFA+CLA, n = 10): 78 g/d linseed (DERBY® Leinöl #4026921003087, DERBY Spezialfutter GmbH, Münster, Germany),4 g/d safflower oil (GEFRO Distelöl, GEFRO Reformversand Frommlet KG, Memmingen, Germany) and 38 g/d Lutalin® (BASF SE, Ludwigshafen, Germany), comprised 0.06 g/d Vitamin E, 2.26 MJ NE_L/d

Acknowledgements

It is my pleasure to acknowledge the role of several individuals who were instrumental for completion of my doctoral research. Foremost, I would like to express my deepest gratitude to my advisor **Dr. Jens Vanselow** for his constant guidance, encouragement and advice that he has provided throughout my time as his student. I also thank him for encouraging the use of correct grammar and consistent notation in my writings and for carefully reading and commenting on countless revisions of the published manuscripts and the dissertation.

This thesis would have not materialized without the financial support of the **FITCOW** project headed by **Dr. Harald Hammon from Institute of Nutritional Physiology "*Oskar Kellner*", FBN**. I am also grateful to the partial funding received through **Federal Ministry of Food and Agriculture (BMEL)** during my doctorate. I wish to convey my gratitude to **Dr. Jennifer Schoen** for her extended discussions and valuable suggestions which contributed greatly to the improvement of my research skills.

The contribution from 'Tiertechnikum" is truly appreciated. I am deeply grateful to our vetenarian team from Tiertechnikum, **Dr. Frank Becker** and **Dr. Volker Röttgen** for conducting rigorous *in vivo* animal experiments. I truly appreciate the valuable inputs of **Dr.Vijay Simha Baddela**, who contributed to many discussions that helped to shape this project along with **Dr. Anya Baufeld** who was always willing to help to resolve on difficult analytical issues.

The fatty acid studies discussed in this dissertation would have not been possible without the necessary facilities provided by **Dr. Dirk Dannenberger** at the **Institute of muscle biology, FBN**. I really appreciate their collaboration and the impressive gas chromatography analysis performed by their technical staff, **Bergit Jentz and Maria Dam.**

I extend my sincere thanks to our laboratory technical staff, **Veronika Schreiter** and **Maren Anders** who always made sure for timely supply of biological samples and gave access to lab equipment's and research facilities. Without their precious support it would have not been possible to conduct this research. I also convey my thanks to **Dr. Torsten Viergutz** and **Christian Plinski** for their technical support in flow cytometry analysis, **Swanhild Rodewald** for carrying out frequent hormone estimation and **Bianka Dwaert** for assisting me in IVF technique.

Last but not the least my deepest appreciation belongs to my source of strength, my family for their unwavering patience and faith in me. Thank you !

Declaration of contribution in each publication

I hereby declare that my contribution in each publication summarized in this dissertation is as follows:

Publication I

Elevated free fatty acids affect bovine granulosa cell function: a molecular cue for compromised reproduction during negative energy balance.

Sharma A, Baddela V.S, Becker F, Dannenberger D, Viergutz T, Vanselow J. *Endocrine connections* (2019), 8(5): 493–505. (doi: 10.1530/EC-19-0011)

- Carried out cell culture experiments
- Preparation of BSA-fatty acid conjugates
- Gene expression analysis
- Statistical evaluation of all gene expression data
- Discussion and interpretation of the data
- Writing the manuscript

Publication II

Effects of dietary fatty acids on bovine oocyte competence and granulosa cells.

Sharma A, Baddela V.S, Roettgen V, Vernunft A, Viergutz T, Dannenberger D, Hammon H.M, Schoen J, Vanselow J. *Frontiers in Endocrinology* (2020),11:87 (doi: 10.3389/fendo.2020.00087)

- Ovary collection and processing
- Carried out cell culture experiments and *in vitro* embryo production (IVP) technique
- Gene expression analysis
- Statistical evaluation of the data
- Discussion and interpretation of the data
- Writing the manuscript

Publication III

Non-Esterified Fatty Acids in the Ovary: Friends or Foes? *Reproductive Biology and Endocrinology* (2020). (**Accepted on 25.05.2020 for publication**)
Baddela V.S, **Sharma A** and Vanselow J

- Literature research and writing the article
- Creation of tables and scientific illustrations

Publication IV

Low oxygen levels induce early luteinization associated changes in bovine granulosa cells.
Baddela VS, **Sharma A**, Viergutz T, Koczan D, Vanselow J. *Frontiers in Physiology* (2018), 9:1066. (doi: 10.3389/fphys.2018.01066)

- Carried out cell culture experiments
- Gene expression analysis

Publication V

HIF1 driven transcriptional activity regulates steroidogenesis and proliferation of bovine granulosa cells.
Baddela V.S, **Sharma A**, Michaelis M, Vanselow J. *Scientific Reports* (2020), 10:3906 (doi:10.1038/s41598-020-60935-1)

- Carried out cell culture experiments
- Gene expression analysis

Arpna Sharma Supervisor: PD Dr. Jens Vanselow

Declaration:

I hereby, declare under oath that i have completed the work submitted here independently and have composed it without outside assistance. Furthermore, i have not used anything other than the resources and sources stated and where i have taken sections from these works in terms of content or text, i have identified this appropriately.

Arpna Sharma