Institut für Tierwissenschaften

Transcription factors mediated oxidative stress response in bovine follicular cells and preimplantation embryos

D i s s e r t a t i o n

zur

Erlangung des Grades

Doktor der Agrarwissenschaft (Dr. agr.)

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich–Wilhelms–Universität Bonn

von

Mohamed Omar Taqi Aboubakr

aus

Kairo, Ägypten

Bonn, 2019

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

Dedicated to my parents, lovely sisters and my cousin

Mohamed Saad

Transcription factors mediated oxidative stress response in bovine follicular cells and preimplantation embryos

Transcription factors (TFs) are DNA-binding proteins implicated in regulation of gene activity and subsequently several biological functions under physiological, pathological and stress conditions. Oxidative stress can be generated naturally or upon exposure to environmental factors that ultimately affect cattle fertility. However, the role and regulation of TFs mediated-oxidative stress response in bovine follicular cells and preimplantation embryos remain elusive. Therefore, this dissertation aims to elucidate the expression pattern, crosstalk, and extracellularly released-TFs under stress condition. To address that, three independent experiments were conducted. In the first experiment, the sub-confluent bovine granulosa cells were treated with H_2O_2 to induce oxidative stress. In the second experiment, NRF2 was selectively knocked down using siRNA. Cells of both experiments were subjected to cellular phenotypes in addition to expression pattern analysis of TFs, endoplasmic reticulum, differentiation and apoptosis genes. In the third experiment, the male and female zygotes were cultured until blastocyst stage under 5% or 20% oxygen levels. Blastocysts were subjected to reactive oxygen species (ROS) level, total cell count assays and mRNA quantification for candidate TFs, antioxidants as well as extracellular vesicles' (EVs) biogenesis and secretion genes. Thereafter, EVs were isolated from spent culture media of embryos and subjected to mRNA quantification of candidate TFs. Results revealed that oxidative stress increased intercellular ROS levels and reduced mitochondrial activity accompanied by lipids accumulation and DNA fragmentation in granulosa cells. Consequently, oxidative stress-induced NRF2 was coupled with differentially expressed candidate TFs associated with a reduction of cell proliferation. Intriguingly, inhibition of NRF2 resulted in upregulation of KLF4 and its target genes, which in turn reduced cellular proliferation. Likewise, high oxygen tension reduced blastocyst rate, total cell number and increased ROS levels in both male and female embryos. Contrary to female embryos, the mRNA expression level of NRF2, KLF4 and SREBF2 was upregulated in male embryos under high oxygen level. Concomitantly, released EVs packed with higher mRNA levels of NRF2 and NOTCH. In conclusion, bovine granulosa cells and preimplantation embryos respond to environmental stressors by differential regulation of TFs.

Transkriptionsfaktoren vermittelte oxidative Stressantwort in Rinderfollikelzellen und Präimplantationsembryonen

Transkriptionsfaktoren (TFs) sind DNA-bindende Proteine, die an der Regulation der Genaktivität und anschließend an verschiedenen biologischen Funktionen unter physiologischen-, pathologischen- und Stressbedingungen beteiligt sind. Oxidativer Stress kann auf natürliche Weise oder durch Einwirkung von Umweltfaktoren erzeugt werden, die letztendlich die Fruchtbarkeit von Rindern beeinflussen. Die Rolle und Regulation der durch TFs vermittelten oxidativen Stressantwort in Rinderfollikelzellen und Präimplantationsembryonen ist jedoch nach wie vor unklar. Ziel dieser Dissertation ist es daher, das Expressionsmuster, das Zusammenwirken und die extrazellulär freigesetzten TFs unter Stressbedingungen aufzuklären. Um dies zu untersuchen, wurden drei unabhängige Experimente durchgeführt. Im ersten Experiment wurden die subkonfluenten Rindergranulosazellen mit H_2O_2 behandelt, um oxidativen Stress zu induzieren. Im zweiten Versuch wurde NRF2 mittels siRNA selektiv ausgeschaltet. Zusätzlich zur Expressionsanalyse von TFs-, endoplasmatischem Retikulum-, Differenzierungs- und Apoptosegenen wurden die zellulären Phänotypen der Zellen beider Experimente bestimmt. Im dritten Experiment wurden die männlichen und weiblichen Zygoten bis zum Blastozystenstadium unter 5% oder 20% Sauerstoffgehalten kultiviert. Die Blastozysten wurden auf ihren Gehalt an reaktiven Sauerstoffspezies (ROS) und die Gesamtzellzahl untersucht und mittels mRNA-Quantifizierung von TFs-, Antioxidantien sowie Biogenese- und Sekretionsgene extrazellulärer Vesikel (EVs) auf ihre Expression analysiert. Danach wurden EVs aus benutzen Kulturmedien von Embryonen isoliert und einer mRNA-Quantifizierung mit TF Kandidatengenen unterzogen. Die Ergebnisse zeigten, dass oxidativer Stress den interzellulären ROS-Spiegel erhöhte und die mitochondriale Aktivität verringerte, begleitet von Lipidakkumulation und DNA-Fragmentierung in Granulosazellen. Folglich wurde durch oxidativen Stress induziertes NRF2 mit differentiell exprimierten TF Kandidatengenen gekoppelt, die mit einer Verringerung der Zellproliferation verbunden waren. Interessanterweise führte die Hemmung von NRF2 zu einer Hochregulation von KLF4 und seinen Zielgenen, was wiederum die Zellproliferation verringerte. Ebenso verringerte eine hohe Sauerstoffkonzentration die Blastozystenrate und die Gesamtzellzahl und erhöhte die ROS-Werte sowohl bei männlichen als auch bei weiblichen Embryonen. Im Gegensatz zu weiblichen Embryonen war das mRNA-Expressionsniveau von NRF2, KLF4 und SREBF2 in männlichen Embryonen unter hohem Sauerstoffgehalt hochreguliert. Gleichzeitig enthielten die freigesetzten EVs einen höheren mRNA-Spiegel von NRF2 und NOTCH. Zusammenfassend lässt sich sagen, dass Rindergranulosazellen und Präimplantationsembryonen auf Umweltstressoren durch unterschiedliche Regulation von TFs reagieren.

Table of contents

List of abbreviations

$^{\bullet}$OH	Hydroxyl radical
18S	18S ribosomal RNA
Abs	Apoptotic bodies
ACTB	Actin beta
AKT	AKT serine/threonine kinase
AlIX	ALG-2 interacting protein X
ANOVA	Analysis of variance
AP-1	Activator protein-1
ARE	Antioxidant response element
ART	Assisted reproductive technologies
ASH	Alcoholic steatohepatitis
ASK1	Apoptosis signal-regulating kinase 1
ATF2	Activating transcription factor 2
Bach1	BTB domain and CNC homolog 1
BAX	BCL2 associated X, apoptosis regulator
BCL2L1	BCL2 like 1
bGCs	Bovine granulosa cells
BSA	Bovine serum albumin
BSA-ITS	BSA and a mixture of insulin, transferrin and selenium
Btk	Bruton's tyrosine kinase
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CAT	Catalase
CCNB1	Cyclin B1
CCND1	Cyclin D1
CCND2	Cyclin D2
CD63	CD63 molecule
CDKN1A	Cyclin dependent kinase inhibitor 1A
cDNA	Complementary DANN
c-FOS	Fos proto-oncogene, AP-1 transcription factor subunit
c-Jun	AP-1 transcription factor subunit
CL	Corpus luteum
CO_2	Carbon dioxide

COCs	Cumulus oocyte complexes
CREB	cAMP responsive element binding protein
Ct	Threshold cycle
CYCS	Cytochrome c
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1
DAPI	4',6-Diamidin-2'-phenylindoldihydrochlorid
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
E2	Estradiol
E2F1	E2F transcription factor 1
E2F4	E2F transcription factor 4
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Elk1	ETS transcription factor ELK1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
ESCRT	Endosomal Sorting Complex Required for Transport
EVs	Extracellular vesicles
EXOs	Exosomes
FBS	Fetal bovine serum
FCS	Fetal calf serum
FOSB	FosB proto-oncogene, AP-1 transcription factor subunit
FOXO1	Forkhead box protein O1
FOXO2	Forkhead box protein O2
FSH	Follicle-stimulating hormone
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA binding protein 4
GnRH	Gonadotropin-releasing hormone
GPBS	Glycine in PBS

GPX	Glutathione peroxidase
Grp78	Glucose-regulated protein 78
Grp94	Glucose-regulated protein 94
GSR	Glutathione reductase
H2DCFDA	6-carboxy- 2',7'-dichlorodihydrofluorescein diacetate
hCG	Human chorionic gonadotropin
HeLa	Henrietta Lacks
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
HIF1	Hypoxia-inducible factor 1
HMOX1	Heme oxygenase 1
$HO_2^{\cdot}$	Hydroperoxyl
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HSC70	Heat shock protein family A (Hsp70) member 8
HSF1	Heat shock transcription factor 1
HSP72	Heat shock protein 72
HSPA8	Heat shock protein A8
IGF-I	Insulin-like growth factor-I
INHBa	Inhibin subunit beta A
INSIG1	Insulin-induced gene 1
IRF7	Interferon regulatory factor 7
IVF	*In vitro* fertilization
IVP	*In vitro* production
JNK	c-Jun NH2-terminal kinase
KDa	Kilo Dalton
KEAP1	Kelch-like ECH associated protein 1
KLF13	Krüppel-like factor 13
KLF2	Krüppel-like factor 2
KLF4	Krüppel-like family 4
KLF6	Krüppel-like family 6
KLF9	Krüppel-like family 9
LH	Luteinizing hormone
Mam	Mastermind
MAPK	Mitogen-activated protein kinase

MAPKK	MAPK kinase
mdm2	Mouse double minute 2
miRNA	Micro RNAs
MKPs	MAP kinase phosphatase
MVBs	Multivesicular bodies
MVs	Microvesicles
NaCHO3	Sodium bicarbonate
NaCl	Sodium chloride
NANOG	Nanog homeobox
NFκB	Nuclear factor-kappa B
NICD	NOTCH intracellular domain
NOTCH1	Notch receptor 1
NOTCH1-IC	NOTCH1 intracellular domain
NRF2	Nuclear factor, erythroid 2 like 2 (NFE2L2)
NTA	Nanoparticle tracking analysis
$O_2^{\bullet-}$	Superoxide anion
OCS	Oestrus cow serum
OD	Optical density
OS	Oxidative stress
p38	Mitogen-activated protein kinase 14
p53	Tumor protein p53
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS-CMF	Ca^{2+}/Mg^{2+} free 1x phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PDCD6IP	Programmed cell death 6 interacting protein
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphatidylinositol 3-kinase
PLB	Passive lysis buffer
POU5F1	POU class 5 homeobox 1
PRDX1	Peroxiredoxin 1

PTEN	Phosphatase and tensin homolog
PVA	Polyvinyl acetate
qRT-PCR	Quantitative real-time polymerase chain reaction
RAB11FIP1	RAB11 family interacting protein 2
RAB27A	RAB27A, member RAS oncogene family
RAB35	RAB35, member RAS oncogene family
RB	Retinoblastoma protein
RNA	Ribonucleic acid
RO˙	Alkoxyl
ROO˙	Peroxyl
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
SEK1	Mitogen-activated protein kinase kinase 4
SEM	Standard error of the mean
siRNA	Small interference RNA
SOD1	Superoxide dismutase 1
SOF	Synthetic oviductal fluid
SOX2	SRY-box 2
SP1	Sp1 transcription factor
SRE	Sterol regulatory element
SREBF1	Sterol regulatory element-binding transcription factor 1
SREBF2	Sterol regulatory element-binding transcription factor 2
STAR	Steroidogenic acute regulatory protein
Syk	Spleen tyrosine kinase
TBST	Tris-buffer saline with Tween 20
TCM	Tissue culture medium
TEM	Transmission electron microscopy
TFs	Transcription factors
TGFβ	Transforming growth factor-beta
TNFα	Tumor necrosis factor-alpha
TSAP6	STEAP3 metalloreductase

TXN1	Thioredoxin
VDAC1	voltage-dependent anion channel 1
VPS4B	Vacuolar protein sorting 4 homolog B
WNT	Wingless signaling pathway
XCI	X-chromosome inactivation

List of Figures

List of Tables

Chapter 1: General overview

1.1 Introduction

Fertility is a multi-factorial and complex trait, therefore, is still not defined well (Walsh et al. 2011). Genetics, management and environment are main factors affecting animal fertility (Kumar et al. 2016; Mathevon et al. 1998; Walsh et al. 2011). In this regard, different types of stressors such as heat stress (Roth 2017) are known to affect animal fertility by disturbing the balance between reactive oxygen species (ROS) and antioxidants. The status in which ROS accumulation exceeds the scavenging ability of antioxidants is termed as oxidative stress. Even though ROS are important in various reproductive functions, it is believed that over-accumulation of ROS has unfavorable effects on folliculogenesis, oocyte maturation and fertilization as well as embryo development (Agarwal et al. 2012; Nishihara et al. 2018). Moreover, the oxidative stress phenomenon could also arise even under controlled systems including *in vitro* embryo production, via culture media, visible light, and oxygen level (Cagnone and Sirard 2016), which leads to decline in quantity as well as quality of *in vitro* produced embryos compared to their *in vivo* counterparts (Farin and Farin 1995; Gad et al. 2012; Pontes et al. 2009; Salilew-Wondim et al. 2015).

Cells respond to stress by modulating the expression of several transcription factors (TFs), which are involved in several biological functions, in order to deal with stress factors (Gad et al. 2012; Leite et al. 2017; Vanselow et al. 2016; Weng et al. 2016). Among those TFs, nuclear factor (erythroid-derived 2)-like 2 (NEF2L2 or NRF2) is essential for activating the battery of antioxidant genes associated with the survival of *in vitro* produced embryos (Amin et al. 2014). Embryos of different sex are known to differ in their transcriptome (Bermejo-Alvarez et al. 2010), epigenome (Bermejo-Alvarez et al. 2008), and metabolome (Gómez et al. 2018). Based on this, we postulate that there may be sexual dimorphic response of embryos to environmental stressors.

The cellular response to stress could be mediated by extracellular vesicles (EVs), which contribute in cell-cell communication (Mathivanan et al. 2010) and elimination of undesirable components from the cell (Takahashi et al. 2017). Despite the small size of released EVs, they carry biological molecules including; DNA, coding and non-coding RNAs, lipids and proteins (Hessvik and Llorente 2018). The transfer of these molecules could alter the biological functions of the recipient cells (Bewicke-Copley et al. 2017; Saeed-Zidane et al. 2017). Interestingly, the component and concentration of EVs cargo could be differed according to the physiological status of the donor cell (Hung et al. 2017;

Navakanitworakul et al. 2016). Accordingly, several investigations were established to address their role in different biological fluids including those related to reproduction. Therefore, a synopsis of the main points mentioned above is discussed in the following points.

1.1.1   Oxidative stress

ROS serve as a double-edged sword in physiological functions, where they are involved as a second messenger in biological pathways (Di Meo et al. 2016; Lu et al. 2018). ROS are produced from different cellular compartments including the plasma membrane, mitochondria, endoplasmic reticulum, peroxisome (Figure 1.1) as byproducts during normal aerobic metabolism processes. In mitochondria, the formation of ROS depends on electron transfer in order to trigger the production of ATPs. As roughly estimated, 2% of consumed oxygen in the cells is converted to ROS during the metabolism process in the mitochondria (Fujii et al. 2005). The most biologically active ROS include hydroxyl radical ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), and superoxide anion ($O_2^{\bullet-}$). However, the hydrogen peroxide (H_2O_2), which can be produced through the reaction of $O_2^{\bullet-}$ with itself and is relatively stable, is not classified as a free radical, but it has the ability to penetrate biological membranes and subsequently breaks down into $^{\bullet}OH$ (Lu et al. 2018). Those reactive species are characterized with instability and highly reactivity till acquiring electrons from the nearby bioactive molecules such as lipids, proteins, carbohydrates, nucleic acids or other molecules, resulting in cellular components damage (Askoxylaki et al. 2013; Lu et al. 2018).

Under normal conditions, there are two types of antioxidants that keep the level of ROS under threshold including enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants comprise superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GSR). On the other hand, non-enzymatic antioxidants include vitamin E, vitamin C, β-carotene, zinc, glutathione, selenium, and taurine (Diamanti-Kandarakis et al. 2017). Initially, SOD stimulates dismutation of $O_2^{\bullet-}$, which is deemed the most detrimental intracellular ROS type and the precursor for other types, to produce oxygen and H_2O_2 (Wang et al. 2017). Subsequently, the latter is removed from the cell by CAT or GPX (Lu et al. 2018; Wang et al. 2017). The GPX is

found to be localized in cytoplasm and mitochondria, whereas CAT is only abundant in peroxisomes (Wang et al. 2017).
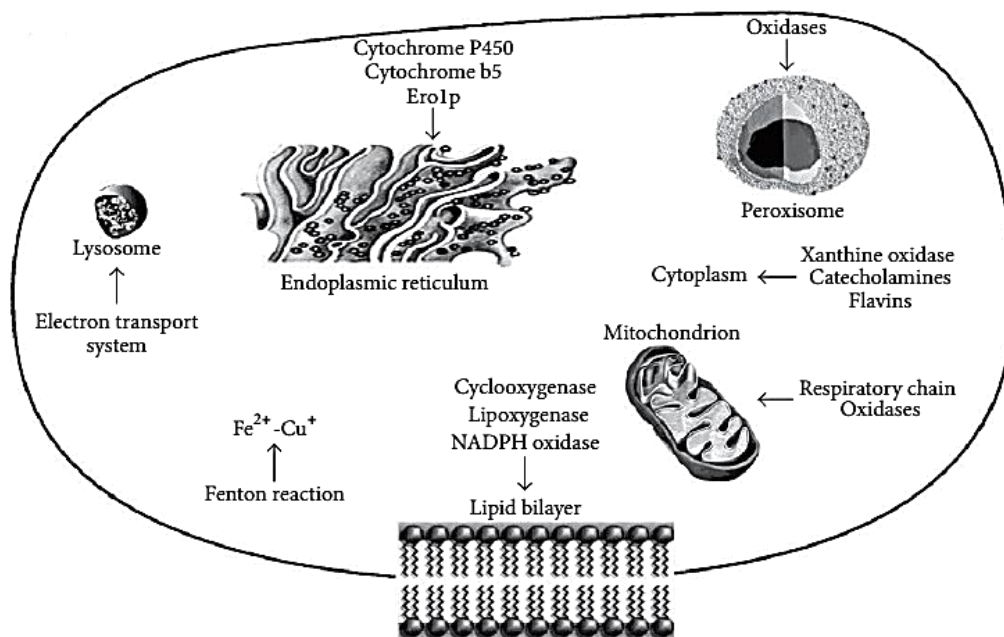
Figure 1.1. Various cellular compartments that are involved in reactive oxygen species production (Di Meo et al. 2016)

However, the disturbance of the balance between ROS production and their scavenger via antioxidants leads to oxidative stress phenomena (Agarwal et al. 2006). This imbalance could arise from endogenous sources such as failure in mitochondrial or endoplasmic reticulum function, which trigger intracellular ROS production dysfunction (Di Meo et al. 2016). Furthermore, it could be exogenously generated from exposure to, for instance, heat stress (Alemu et al. 2018; Cong et al. 2017), heavy metals (Wang et al. 2011) or radiation (Chen et al. 2019). Indeed, the effect of excessive levels of intracellular ROS ranges from stimulation (Shkolnik et al. 2011; Vandaele et al. 2010) to harmful (Yang et al. 2017) for biological functions, which indicates that their effects are level-dependent. Most of previous results were focused on the deleterious effect of ROS and showed that the overproduction of ROS is harmful to biological functions, which are represented in, for example, interaction with biomolecules and subsequently damage nucleic acids (DNA and RNA), proteins and lipids (Martindale and Holbrook 2002; Willi et al. 2018). Consequently, the oxidative stress could induce cell cycle arrest, alteration in chromosomal alignment, compromise the mitochondrial activity and integrity as well as cell membrane (Kadenbach et al. 2004; Kala et al. 2017; Saeed-Zidane et al. 2017; Sohel

et al. 2019; Zhang et al. 2016), which could eventually lead to increase apoptosis (Sohel et al. 2019; Yang et al. 2017; Zhang et al. 2016). In addition to this, the induction of oxidative stress could disturb the protein biosynthesis through induction of endoplasmic reticulum stress (Gao et al. 2012). This combination of two types of stress may be more harmful to the physiological functions and increase the sensitivity to other stressors. Therefore, cells are well equipped by several defense mechanisms in order to cope with the stress and acquire the adaptive response, which are the focus of the review below.

1.1.2   Association between oxidative stress and other stressors

Adaptation of living organism to either internal or external stimuli is necessary for maintaining different physiological functions (Scandalios 2005). The exposure of animals to variety of changes including climate, transportation, feeding, husbandry, and prophylactic therapy may result in multifactorial stress (Figure 1.2) (Chen et al. 2015). These stressors may negatively affect animal health and productivity (Rahal et al. 2014). In a dairy herd, sickness could occur at any time. However, the animals are most susceptible to diseases during either periparturient or neonatal periods (Sordillo and Aitken 2009). During the last decade, many research works revealed that mature (especially around calving time) and/or immature (represented the first few weeks of neonatal) animals experience oxidative stress (Abuelo et al. 2014). At the time of oxidative stress, the immune status of the animal is very weak, leading to increasing the chance of disease susceptibility (Figure 1.3) (Abuelo et al. 2019). Dairy cows could be susceptible to metabolic stress when they fail to adapt to high nutrition requirement that are necessary for lactogenesis as well as fetal growth, and parturition (Baumgard et al. 2017). In ruminants, the influence of oxidative stress in animal health during the dry period is very critical to animal productivity as well as reproduction (Abuelo et al. 2019). Li and his colleagues reported that the exposure of cells to intense heat stress resulted in releasing of ROS and subsequently activate the apoptotic pathway in mitochondria (Li et al. 2017).
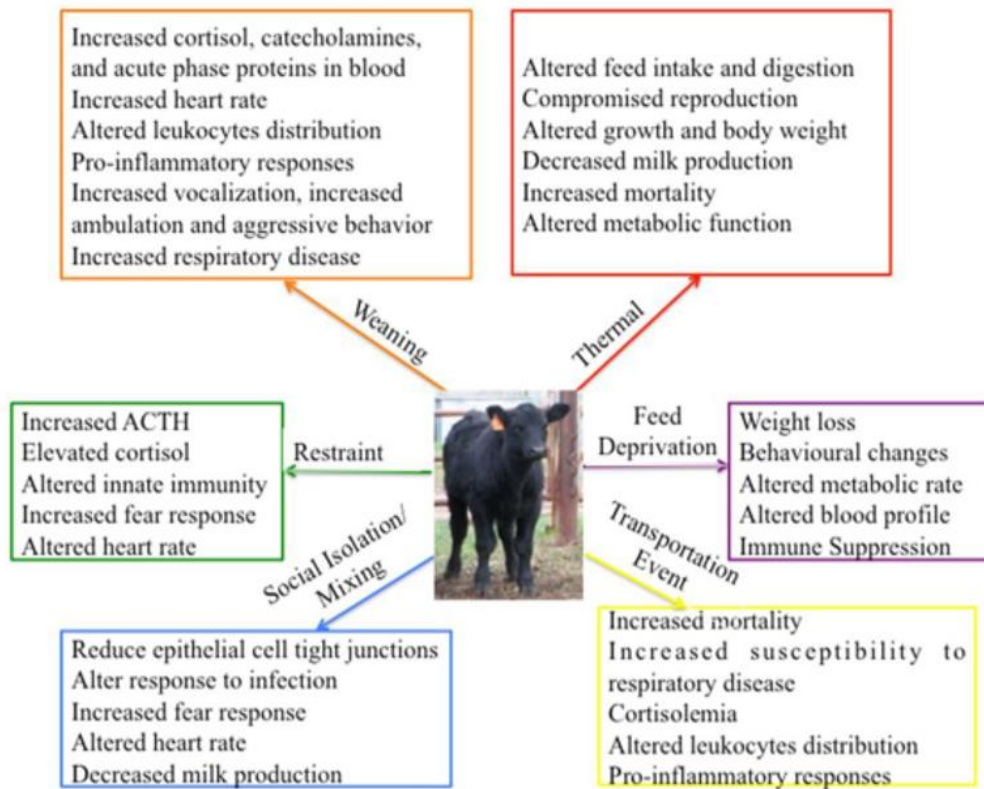
Figure 1.2. Different sources of stressors and their effect on mammalian health and productivity, this figure is adopted from Chen et al (2015)
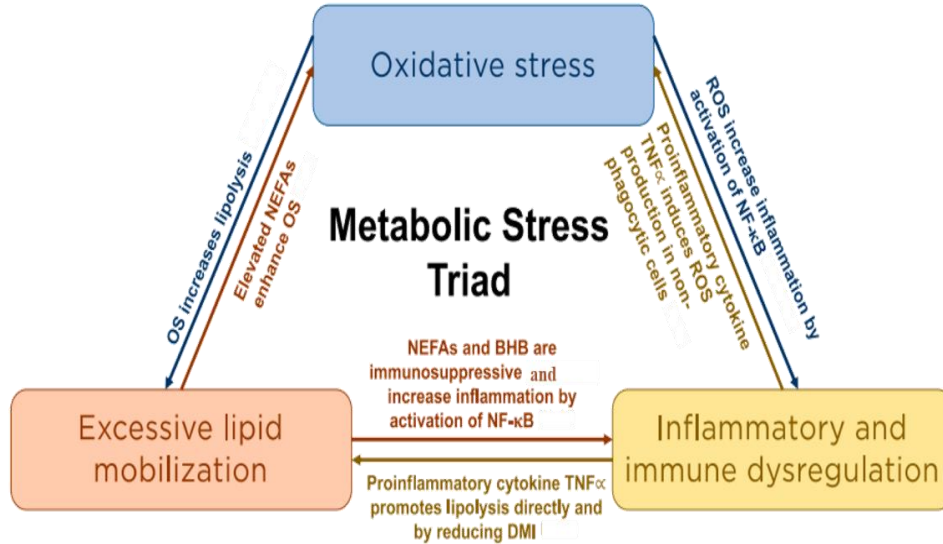
Figure 1.3. Schematic diagram of the intrinsic relationships among the various components of the metabolic stress triad. ROS: Reactive oxygen species; NFκB: nuclear factor kappa-B; NEFA: non-esterified fatty acid; OS: Oxidative Stress; BHB: beta-hydroxybutyrate; TNFα: Tumor necrosis factor-alpha; DMI: Dry matter intake. This figure is adopted from Abuelo et al (2019)

As previously discussed, the ROS synthesis can be induced as a result of different environmental stress conditions such as; exposure to high temperature, different toxins, UV, heavy metals pollution, herbicides, and xenobiotics. Ample evidence revealed that many oxidative stress inducers are harmful, and lead to mutations as well as cancers (Abuelo et al. 2019; Godet et al. 2019). The accumulation of ROS is a predisposing factor for many diseases and environmental issues (Scandalios 2005).

1.1.3   Cellular defense mechanisms against oxidative stress

1.1.3.1   NRF2 signaling pathway

NRF2 is a master TF of oxidative stress response in the cell (Itoh et al. 1999; Li et al. 2004), and regulates many antioxidant transcripts (Nguyen et al. 2009), especially genes coding for antioxidant enzymes such as PRDX1, SOD1, GPX4, TXNRD1 (Amin et al. 2014; Gad et al. 2012), as well as detoxifying, stress response, and proteasomal degradation-related genes. The NRF2 is a nuclear protein with half-life ~15 min and becomes transcriptionally active under both constitutive and stress conditions (Nguyen et al. 2009). In case of constitutive conditions, NRF2 is in a sequestered form in cytoplasm by Kelch-like-ECH-associated protein 1 (KEAP1) and targeted to proteasomal degradation (Itoh et al. 1999; Wakabayashi et al. 2003). On the other hand, under oxidative stress conditions, NRF2-KEAP1 interaction is disrupted in a dose-dependent manner (Amin et al. 2014; Itoh et al. 1999), which leads to a translocation of NRF2 to the nucleus, in order to activate the transcription of antioxidant and detoxifying genes by binding to the regulatory region of antioxidant response elements (AREs) as has been shown in Figure 1.4 (Itoh et al. 1999; Namani et al. 2014). Consequently, the detrimental levels of intracellular ROS are reduced under threshold levels to confer cell survival. Supporting this notion, the survival of bovine embryos exposed to oxidative stress is associated with the ability of the embryo to activate the NRF2-signaling pathway (Amin et al. 2014). In response to over-accumulation of ROS in the nucleus, the NRF2 can promote apoptosis through binding and activating krüppel like factor 9 (KLF9) and consequently leading to increase in ROS levels (Zucker et al. 2014). On the other hand, the transcription factor BTB domain and CNC homolog 1 (Bach1) has been shown to suppress the induction of several NRF2 target genes via interaction with the antioxidant response element (ARE) sites in their promoters (Sun et al. 2002).
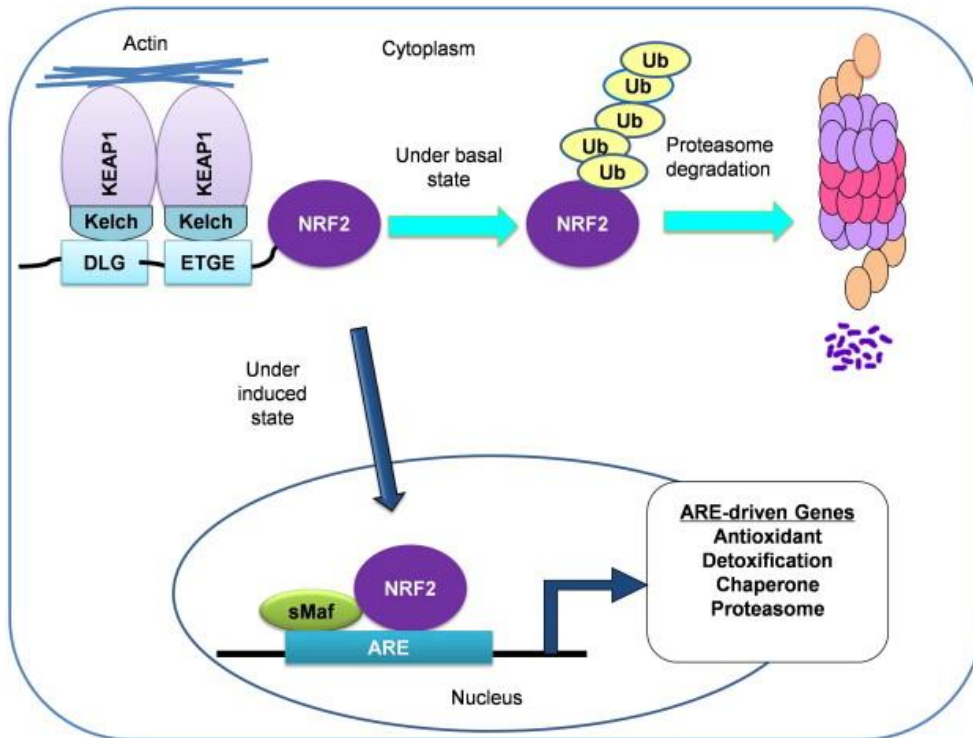
Figure 1.4. Schematic model of the NRF2–KEAP1 signaling pathway under basal and stress conditions. NRF2 indicates nuclear factor (erythroid-derived 2)-like 2, KEAP1 indicates Kelch-like-ECH-associated protein 1, ETGE and DLG are KEAP1 motifs, ub is ubiquitination that occurs to NRF2 during the degradation process, sMaf is small Maf proteins that bind to NRF2 in order to activation antioxidant response element (ARE). This figure is adopted from Namani et al (2014)

1.1.3.2   Mitogen-activated protein kinase pathway

Mitogen-activated protein kinase (MAPK) encompass a large number of serine/threonine kinases involved in the regulation of cell proliferation, differentiation, stress adaptation, and apoptosis (Son et al. 2011). This pathway may play an essential role in transduction signaling cascades from the cell membrane to the nucleus via several phosphorylation events (Brown and Sacks 2009). Upon MAPK pathway activation, it phosphorylates various substrate proteins including several TFs such as Jun proto-oncogene, AP-1 transcription factor subunit (c-Jun), activating transcription factor 2 (ATF2), ETS transcription factor ELK1 (Elk1), and tumor protein p53 (p53) (Kim and Choi 2010). They are divided into three major subfamilies: the extracellular signal-regulated kinases (ERK), the mitogen-activated protein kinase 14 (p38) and the c-Jun N-terminal kinases

(JNK; as stress-activated protein kinase or SAPK). Those subfamilies could be activated independently via signaling pathway involved MAPK kinase (MAPKK) (Figure 1.5) (Kim and Choi 2010; Martindale and Holbrook 2002). This pathway could be activated via induction of oxidative stress. Supporting this notion, exposure of human keratinocytes to H_2O_2-induced oxidative stress led to activation of epidermal growth factor receptor (EGFR) through phosphorylation of receptor tyrosine kinases (RTK) (Meves et al. 2001). The EGFR was found to activate the ERK pathway via insulin-like growth factor-I (IGF-I), which could be disturbed by inhibition of EGFR indicating that the activation of the ERK pathway relies on EGFR activation. The ROS production was triggered by IGF-1 and antioxidants suppressed the activation of EGFR via IGF-1 indicated that ROS mediate the activation of the ERK pathway through IGF-1 (Meng et al. 2007). Furthermore, ROS-induced MAPK pathway has also occurred via oxidative modification of MAP3Ks.

Apoptosis signal-regulating kinase 1 (ASK1), which is a MAP3K member and regulated via ROS and tumor necrosis factor-alpha (TNFα), mediates the activation of JNK and p38 pathways in response to oxidative stress (Nagai et al. 2007). ROS might inactivate and degrade the MAP kinase phosphatase (MKPs), which contributes to maintaining the pathway in an inactive form (Son et al. 2011). Consistently, higher levels of intracellular H_2O_2 resulted in inhibition of MKPs, which in turn leads to activation of JNK pathway that is needed for releasing of cytochrome c and caspase 3 cleavage thereby induce apoptosis (Kamata et al. 2005). Similarly, the JNK and p38 genes were upregulated in response to perfluorooctane sulfonate-induced oxidative stress and subsequently induced cell apoptosis in zebrafish larvae (Shi and Zhou 2010). Under the inflammatory condition, the expression of p38 MAPK and JNK was upregulated, which could activate nuclear factor kappa B (NFκB) through MAPK-NRF2 signaling pathway (Guo et al. 2015). On the other hand, the H_2O_2-mediated activation of MKP-1 increased the resistance of MCF-7 cells to cell death via suppression of JNK and p38 activity (Zhou et al. 2006). Moreover, the deleterious effect of ROS accumulation was prevented via the treatment of mouse embryonic stem cells with dihydrotestosterone, which in turn inhibited the p38 MAPK triggered by ROS (Lee et al. 2008).
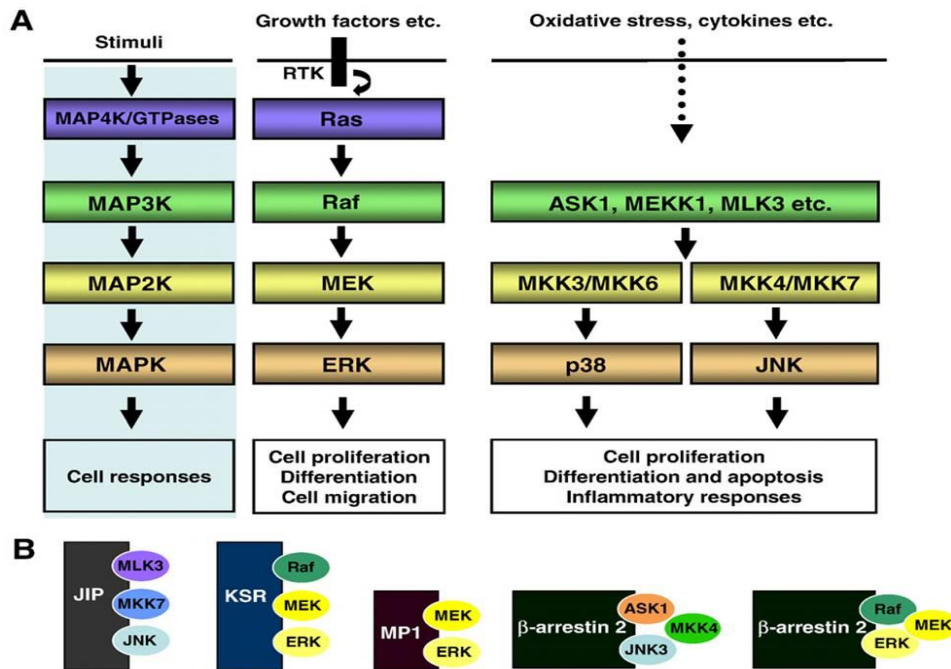
Figure 1.5. A schematic representation of Mitogen-activated protein kinase (MAPK) signaling pathways. (A) This pathway is initiated via extracellular or intracellular stimuli, which in turn leads to activation of MAP kinase kinase kinase (MAP3Ks)-mediated phosphorylation and activation of MAP2Ks and subsequentially activation of MAPKs followed by phosphorylating various substrate proteins including transcription factors, resulting in regulation of a variety of cellular activities including cell proliferation, differentiation, migration, inflammatory responses, and death. Activation by MAPK signaling cascades is accomplished either through a series of binary interactions among the kinase components or through scaffold protein-mediated formation of a multiple kinase complex. (B) Scaffold proteins facilitate activation of MAPK signaling pathways, whereby RTK, receptor tyrosine kinase; ERK, extracellular signal regulated kinase; JNK, c-Jun NH2-terminal kinase; ASK1, apoptosis signal-regulating kinase 1; MLK, mixed-lineage kinase; JIP, JNK-interacting proteins; KSR, Kinase suppressor of Ras-1; MP1, MEK partner 1 (Kim and Choi, 2010)

1.1.3.3 PI3-Kinase/AKT pathway

Phosphatidylinositol 3-kinase (PI3K) and its phospholipid products regulate cell survival from extracellular stimuli, which threaten the cell (Caro and Cederbaum 2006). The

activation and phosphorylation of AKT serine/threonine kinase (AKT) are initiated by converting plasma membrane lipids phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate via PI3K, which binds to AKT and phosphoinositide-dependent kinase 1 (PDK1) and consequently phosphorylate AKT (Lima et al. 2006). AKT provides a survival signal that protects cells from apoptosis induced by oxidative stress or other stresses. This action occurs under the regulation of spleen tyrosine kinase (Syk) and Bruton's tyrosine kinase (Btk), which are essential for AKT survival pathway and enhancing the resistance of cells to oxidative stress-induced apoptosis (Ding et al. 2000; Kikuchi et al. 2011; Takano et al. 2002). Additionally, the EGFR was found to mediate the oxidative stress-induced AKT activation through H_2O_2-induced EGFR phosphorylation (Wang et al. 2000). Therefore, the activation of AKT protects cells against oxidative stress-induced apoptosis via EGFR/PI3K-dependent pathway. Beside EGFR, focal adhesion kinase (FAK) was found to be implicated in the regulation of the PI3K/AKT survival pathway (Sonoda et al. 1999). Consistently, overexpression of FAK activates PI3K/AKT survival pathway and inhibits the activation of caspase-3 protease resulting in cellular survival (Ben Mahdi et al. 2000). Furthermore, phosphorylation of AKT inhibited the expression of ASK1 and subsequently prevented its ability to activate JNK and its downstream target gene ATF2 leading to protection from oxidative stress-induced cell apoptosis (Kim et al. 2001). However, the pretreatment of human luteinized granulosa cells with hCG reduced IGF-1-induced AKT phosphorylation and cell proliferation, not phosphorylation of ERK1/2, which could be regulated by phosphatase and tensin homolog (PTEN) (Goto et al. 2009).

In cattle, the higher phosphorylation of AKT is positively correlated with oocyte competence as well as progesterone secretion and negatively with apoptosis rate of cumulus cells, which indicated its role in oocyte maturation (Salhab et al. 2011). Besides that, there is crosstalk among PI3K/AKT and other pathways to promote cell survival against inflammatory and oxidative damages such as NRF2-signaling pathway (Qi et al. 2012; Xu et al. 2015). However, the inhibition of PI3K/AKT-mediated cell defense mechanism via suppression of AKT phosphorylation inhibited the activation of NRF2/HO-1 expression (Xu et al. 2015), which indicates its vital role in the activation of NRF2-mediated antioxidant response.

1.1.3.4 Heat Shock Proteins (HSPs)

The induction of HSPs in response to stress is mediated by heat shock transcription factor 1 (HSF1). It was found to be stimulated by various stress stimuli such as heat, oxidative stress and protein damage. Antioxidants play a role in cell protection via either directly at the level of HSF1 to modify its activity or prevent HSF1 activation (Martindale and Holbrook 2002). HSPs may confer resistance to oxidative stress, by preserving proteasome, an enzyme responsible for the majority of intracellular proteolysis, function and attenuating the toxicity of proteasome inhibition, which occurs as a result of oxidative stress (Ding and Keller 2001). The protection function of small shock protein occurs through either decreasing the intracellular ROS levels by modulating the metabolism of glutathione to maintain it in a reduced state or inhibit other apoptotic signaling pathways (Martindale and Holbrook 2002). For example, heat shock protein 72 (HSP72) can modulate stress-activated signaling by directly inhibiting JNK, which induced HSP72 production and thereby inhibit JNK-mediated apoptosis (Park et al. 2001). It also may prevent JNK activation by a JNK kinase such as mitogen-activated protein kinase kinase 4 (SEK1). Furthermore, it may accelerate the JNK dephosphorylation (Volloch et al. 2000). On the other hand, the overexpression of HSP70 or heat shock protein family A (Hsp70) member 8 (HSC70) prevents cells from heat shock-induced cell death by inhibiting the processing of procaspases 9 and 3 (Mosser et al. 2000). This redox-dependent activation of HSF1 by heat or H_2O_2 contributes to activation of HSPs gene expression (Ahn and Thiele 2003).

1.1.3.5 p53 signaling pathway

The p53 pathway responds to stressors that can disrupt the DNA replication and cell division (Harris and Levine 2005). Moreover, it plays a vital role in regulating cellular senescence and aging (Gambino et al. 2013). Additionally, it regulates cellular redox state through potential activation of glutathione peroxidase (GPX) (Tan et al. 1999). Accordingly, the imbalance of redox state may influence the downstream biological response of p53 according to stress duration (Martindale and Holbrook 2002). The stress signal is conveyed to p53 protein by post-translational modifications resulted in the p53 activation, which involves in cell cycle arrest, cellular senescence or apoptosis (Gambino et al. 2013; Harris and Levine 2005). Regarding cell cycle arrest, its activation relies on three genes namely; cyclin-dependent kinase inhibitor 1A (p21), 14-3-3σ and growth

arrest and DNA damage-inducible 45 (GADD45). Upon p21 activation, this stimulates G1 cell cycle arrest via suppression of G1 cyclin-dependent kinases (cyclin A/cdk2, cyclin E/cdk2, cyclin D/cdk4 complexes) (Gartel and Tyner 2002; Harper et al. 1993). Additionally, it can stimulate cell cycle arrest at G2/M through GADD45 (Wang et al. 1999). Moreover, p53 could induce cell apoptosis through upregulation of p58, which is involved in the cell death process, in independent PI3K manner (Yin et al. 1998). p53 can also directly activate apoptosis in a transcription-independent manner in MG-63 osteosarcoma cell line (Ferreira de Oliveira et al. 2014). Eventually, this pathway leads to release of cytochrome c from the mitochondrial membrane, which acts as a mediator in apoptosis cascades. On the other hand, p53 itself activates mouse double minute 2 (mdm2) transcription resulting in a negative feedback loop on p53 (Harris and Levine 2005), in order to prevent the accumulation of p53 within the cell (Kruse and Gu 2009). Moreover, the excessive accumulation of p53 within the developing embryo compromises the embryo viability and then implantation (Ganeshan et al. 2010).

Regarding its crosstalk with other pathways, it was found that p53 could be reasonable for sustained activation of MAPK, which inhibited by expression of dominant-negative Ras (N17Ras) and Raf1 mutants (Lee et al. 2000). In primary fetal bovine fibroblasts, oxidative stress negatively impacts their lifespan through regulation of p53, serine 20-p53 phosphorylation and p66^{shc}, which may be involved in senescence (Favetta et al. 2004a). Furthermore, the pro-apoptotic role of p66^{Shc} requires phosphorylation of serine 36 indicating that its effect is independent form (Tiberi et al. 2006). Confirming this, the early arrested bovine embryos during *in vitro* production was associated with higher expression of p66^{sh} (Favetta et al. 2004b). Furthermore, parthenotes exhibited more pronounced pro-apoptotic pattern than *in vitro* produce counterparts, through regulation of p66^{sh} (Gómez et al. 2009). However, its downregulation was concomitant by upregulation of an anti-apoptotic gene, BCL-2, which in turn results in tolerance to oxidative stress (Andoh et al. 2000).

1.1.4 Transcription factors associated with oxidative stress

Cells express arrays of molecules, which are necessary for their stability and precise metabolic function, according to cell type. At the genomic level, cells respond in a precise and prompt manner, either by overexpression or inhibition of the expression of certain genes (Scandalios 2005). Those genes, that are upregulated in response to stress stimuli,

play an important role in maintaining the survival of living organisms (Dalton et al. 1999; Godet et al. 2019; Scandalios 2005). The different regulatory mechanisms that modulate the activity of gene expression includes TFs. The TF are DNA-binding proteins, which are responsible for regulating the activity of gene via binding to a certain region on the DNA (Zabet and Adryan 2013). In mammalian cells, it was recorded that AP-1, NRF2, cAMP responsive element binding protein (CREB), HSF1, hypoxia-inducible factor 1 (HIF1), TP53, NFκB, NOTCH, Sp1 transcription factor (SP1), and SCREB-1 play a role during stress conditions; mainly in response to H_2O_2 stimulation (Marinho et al. 2014). Mammalian cells are well equipped to adapt for any stress at a certain threshold, and in case of increasing the exposure time to stress, this could induce drastic alterations in the regulation of gene activity either at transcriptional or posttranscriptional levels (Godet et al. 2019; Kumar et al. 2012). Under stress conditions, the accumulated ROS cause profound changes in the expression pattern of TFs, as well as stress response genes (Leite et al. 2017). These regulatory mechanisms and their pathways become more complicated with increasing phylogenetic tree (Marinho et al. 2014). For instance, there are multiple sensors as well as pathways involved in response to H_2O_2 stimulation, in order to regulate mammalian cell response during stress condition, through different levels; either (I) synthesis of TF and subsequently upregulation of mRNA stability and translation, (II) maintain the stability of TF, (III) control cytoplasm–nuclear traffic, (IV) regulate DNA binding and nuclear transactivation (Mahadev et al. 2001; Marinho et al. 2014; Woo et al. 2010).

KLF9 is a TF belonging to KLFs family that regulates the intracellular ROS, through binding to the promoter, and then alters the expression of several genes. On the other hand, KLF9 can cause accumulation of intracellular ROS at least in part by transcriptional suppression of the thioredoxin reductase 2 gene (Txnrd2). Txnrd2, a mitochondrial selenoprotein, plays a critical role in defense against oxidative damage and is considered as the major regulator of ROS in mitochondria (Arnér 2009). High dose of sulforaphane (12-14 µM), which is a NRF2 activator, or H_2O_2 resulted in upregulation of KLF9, but the low dose (10 µM) did not induce KLF9 expression. Therefore, KLF9 induction was related to high amounts of NRF2 expression. Accordingly, KLF9 may be expressed in KEAP1 knockout fibroblasts without H_2O_2 treatment and suppressed to the original levels by partial depletion of NRF2 (Zucker et al. 2014). The transcription factor Bach1 suppresses the induction of several NRF2 target genes via interaction with the ARE sites

in their promoters (Sun et al. 2002). In addition to the role of KLF9 in oxidative stress, it may play as a tumor suppressor (Tetreault et al. 2013), through induction of oxidative stress as a mechanism of cell killing to suppress tumor progression (Zucker et al. 2014). Furthermore, oxidative stress could induce the MAPK signaling pathway, and their TFs, such as NRF2 and NFκB. Those TFs could induce expression of pro-inflammatory mediators resulting in inflammation and related diseases. Therefore, silica nanoparticles, which have toxicological effects, was showed to activate the MAPK signaling pathway via phosphorylation of p38 and JNK, which ultimately activate the NFκB via the MAPK-NRF2 signaling pathway (Guo et al. 2015). NFκB is activated via various stimuli leading to phosphorylation of IκB, which is a cytoplasmic inhibitory protein of NFκB preventing its translocation to the nucleus (Martindale and Holbrook 2002). Diverse experimental models indicated the presence of cross-talk between NOTCH1, which is a TF involved in the cell-fate decision, differentiation, proliferation and apoptosis, and NFκB signaling pathways. Oxidative stress-induced alcoholic steatohepatitis (ASH) was found to be mediated by the NOTCH1-NFκB signaling pathway, which could be effectively reversed by inhibiting the NOTCH1 gene (Wang et al. 2014). Furthermore, NOTCH1 intracellular domain (NOTCH1-IC) contributes to the prevention of oxidative stress-induced cell death via suppression of ASK1 and its downstream target genes. On the other hand, it facilitates the translocation of ASK1 toward the nucleus. Therefore, cells-depleted of NOTCH1 gene were more susceptible to oxidative stress-induced apoptosis through activation of ASK1-MKK3/MKK6-p38 MAPK signaling cascade (Mo et al. 2013).

1.1.5   Ovarian functions and oxidative stress

In cows, the estimated reserve of primordial follicles is around 130,000 at birth (Erickson 1966). However, most of these follicles never reach ovulation, whereas more than 99% will undergo atresia (Hsueh et al. 1996). Therefore, assisted reproductive technologies (ART) play an important role in managing and improving reproduction in humans as well as different animal species (Girard et al. 2015). During the different steps in ART, oocytes could face some kind of oxidative stress, which might hamper the competence of early embryonic development (Roth 2017).

In ovarian tissue, ROS play roles during different physiological functions of ovary such as; maturation of oocytes, ovulation process, steroidogenesis, blastocyst formation, implantation, formation, maturation, and regression of corpus luteum (CL), (Lu et al.

2018). It was demonstrated that ROS have a dual effect on mammalian ovarian tissues (Lu et al. 2018; Shkolnik et al. 2011), as shown in table (1). The main sources of ROS in follicular fluid are macrophages and leukocytes (Fujii et al. 2005). It was mentioned that functional granulosa cells produce higher amounts of ROS during the process of electron transport (Du et al. 2006). In patients suffering from polycystic ovary syndrome (PCOS), a reduction of O_2 consumption by mitochondria and induction of ROS production were noticed, which could be the reason for mitochondrial dysfunction (Hilali et al. 2013). Nevertheless, excessive accumulation of endogenous ROS resulted in different cell injuries like DNA damage, protein oxidation, lipid peroxidation, and enzyme inactivation (Shi et al. 2016; Valko et al. 2007). Murdoch (1998) revealed the role of oxidative stress in the induction of follicular atresia through stimulation of apoptosis pathway in granulosa cells (Murdoch 1998) Moreover, the severity of stress could vary according to follicle stage-dependent manner, like the response of different developmental stage of follicles to heat stress (Figure 1.6) (Roth 2017).
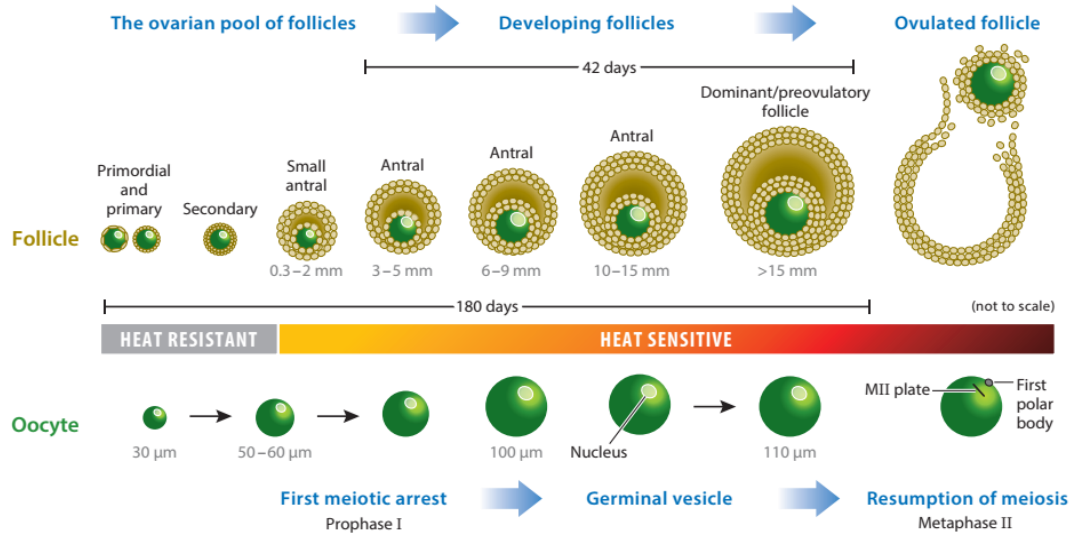
Figure 1.6. Diagram illustrating the resistance/sensitivity of the ovarian follicles and their enclosed oocytes to heat stress in a stage-dependent manner. The primordial, primary, and secondary follicles are heat resistant, whereas the developing antral follicles, including the dominant and preovulatory follicles, are sensitive to heat exposure with a prominent effect on the germinal vesicle–stage oocyte (developing stage) and metaphase II (MII)-stage oocyte (ovulation). Adopted from Roth (2017)

Table 1.1. The role of oxidative stress in the female reproductive process

Function	Reproductive process
Positive effect	Zn-Cu SOD↑ → Promotion of the development of follicles
	Biosynthesis of ovarian steroids → P450↑ → ROS↑ → Blood flow↑ → Rupture of follicles → Ovulation
	ROS↑ → Promotion apoptosis of non-dominant follicles FSH↑ → E2↑ → CAT and GSH↑ → Protection of cells from apoptosis
	E2 and P ↓ → SOD↓ → OS↑ → Endometrial shedding and implant failure
	ROS↑ → NF-κB↑ → $PGF_{2\alpha}$↑ → Luteum dissolution
	Sperm-ovum binding → ROS↑ → Corpus luteus functional↑ ROS↑ → Antioxidants↑ → Synthesis of progesterone
Negative effect	PCOS: Serum proline activity↑, OS↑ Physiological hyperglycemia → ROS↑ (Monocytes) → TNF-α↑ → NF-κB↑ → Resistance of Insulin↑
	Preeclampsia: Defective placenta → Hypoxia and reperfusion injury → OS↑ → Cytokines↑, Prostaglandins↑ → TAS↓, GPx of placenta↓ V_C↓ → Risk of preeclampsia↑ (MDA↑) ROS↑ → Vasoconstriction↑ → Coagulation activity↓ OS↑ → Vascular endothelial injury↑ → ROS↑ → TNF-α↑, ox-LDL↑ → Endothelial subtypes of activated NAD(P)H oxidase → SO anion↑ Auto-antibodies of AT1-AA↑ → NAD(P)H oxidase↑ → ROS↑ → SO anion↑
	Endometriosis: In the peritoneal fluid, MDA↑, IL-6↑,TNF-α↑, IL-8↑, VEGF↑, MCP-1↑, ox-LDL↑. In endometriotic lesions: OS↑ → NF-κB↑ → Inflammation↑ In endometriotic cells: MAPK↑ ROS↑ → IUGR, abortion, fetal malformation

The '→' indicates that it has an effect on the next step. The '↑' represents an increase and the '↓' represents a decrease

Adapted from Lu et al. (2018)

1.1.6   Factors that affect stress susceptibility in preimplantation embryos

The exposure of embryos to different environmental stressors showed a profound effect on the developmental plasticity of embryos (Figure 1.7), which could be traced either at pre- or/and post-implantation stages in a sex-specific manner (Pérez-Cerezales et al. 2018). Male and female embryos exhibit an extensive transcriptional dimorphism, which is mainly driven by incomplete X chromosome inactivation. Therefore, the early stages of embryonic development are very crucial to establish epigenetic regulation in order to be conserved through different development stages (Chang et al. 2019; Pérez-Cerezales et al. 2018). During gonadal formation, there are hormonal differences between male and female placentae, which show different responses to environmental stressors. At the maternal level, the pregnancy itself is considered as a state of oxidative stress, which
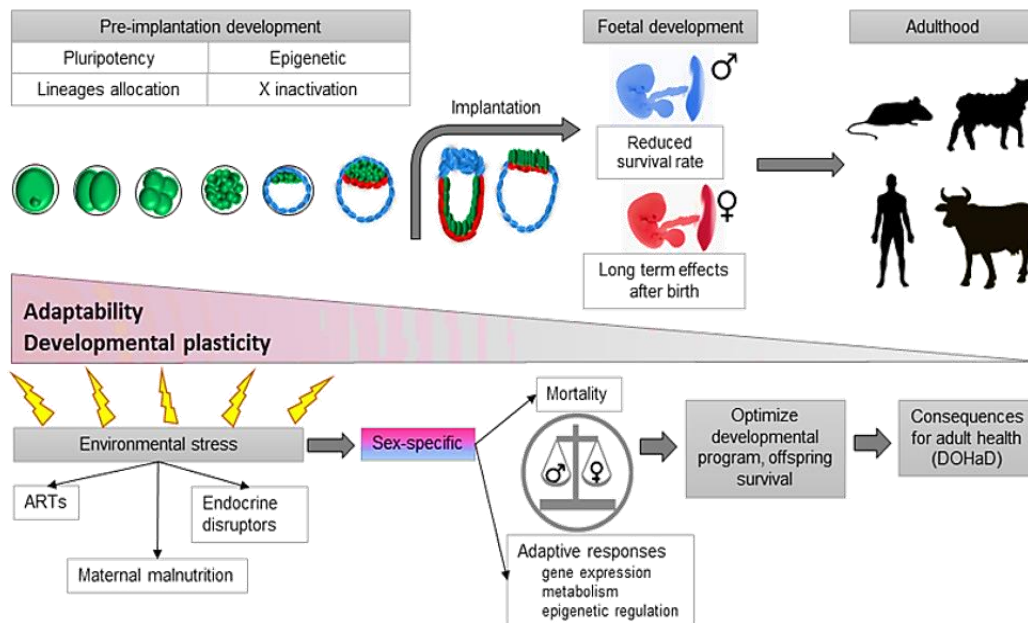
Figure 1.7. Pre- and peri-implantation embryo adaptations to environmental stress. Critical events taking place during this developmental window (pluripotency emergence, epigenetic reprogramming, lineage allocation and X chromosome inactivation) render the embryo especially sensitive to environmental stress. Such embryos and the resulting foetus respond to stressors by sex-specific mortality or by adaptive responses in order to optimize their developmental program and offspring survival. The female placenta (in pink), due to its higher adaptability, buffers more efficiently the impact of endogenous and exogenous stressors on the foetus and it is less compromised than male foetus (in blue) under similar stress conditions. DOHaD indicates Developmental Origins of Health and Disease (Pérez-Cerezales et al. 2018)

resulted from higher metabolic activity in the placental mitochondria, and subsequently associated with the higher release of ROS due to the demand of the growing fetus (Myatt and Cui 2004; Wisdom et al. 1991).

The sex differences in gene expression take place during embryonic genome activation, and they show dynamic patterns throughout the lifespan. However, some studies showed that transcriptional sex differences are higher in adult tissues compared to preimplantation embryos (Lowe et al. 2015). Sex-biased expression of Y- and X-linked genes is not only due to difference between male and female embryos with genes located on sex chromosomes alone but also many autosomal genes (Bermejo-Alvarez et al. 2010; Kobayashi et al. 2006). During early stages of embryonic development, fetuses are highly susceptible to external or internal insults such as oxidative stress either during transcription, replication, or meiotic rearrangement, which will be reflected on tissue as well as organ differentiation (Sundrani et al. 2017). Gametes, which is responsible for transmitting genetic information correctly to the next generation, is prone to remove by apoptosis (Aitken and Baker 2006; Tsunoda et al. 2014). Therefore, identification of relevant sex-dependent stress response pathways is essential for the development of future diagnostic markers (Pérez-Cerezales et al. 2018).

1.1.7   Extracellular vesicles function and composition

In mammals, communication among cells is fundamental for the regulation of different biological functions including proliferation, development, differentiation, stress response, gametogenesis and embryogenesis. It was believed that the communication among cells is limited to endocrine, autocrine, paracrine, cell to cell adhesion, releasing growth factors and neurotransmitters (Perbal 2003; Raposo and Stoorvogel 2013). However, this theory was changed after the discover EVs, which are found for the first time in the human plasma and named as "platelet dust" (Wolf 1967). In 1983, it was observed that the mechanism of releasing these vesicles depends on the fusion of multivesicular bodies (MVBs) to the plasma membrane (Harding et al. 1983). Later, scientists found that these vesicles contain different types of RNA including coding and non-coding RNA (Valadi et al. 2007).

EVs were defined as double-layered membrane vesicles originating from cellular cytosol and released into the extracellular space (Chiang and Chen 2019; Lasser et al. 2011). Their size ranges from 30 to 5000 nm in diameter and their lumen contains various

bioactive molecules such as RNAs, DNA, protein and lipids. EVs play a vital role in cellular function either in physiological (Hung et al. 2017; Valadi et al. 2007) or pathological (An et al. 2015; Anderson et al. 2010) processes. They were found in different biological fluids including plasma, milk, saliva, urine (Lawson et al. 2016), semen ejaculate (Hoog and Lotvall 2015), follicular fluid (Hung et al. 2015; Hung et al. 2017) and uterine aspirates (Campoy et al. 2016). Different morphological characteristics of EVs were found in human ejaculate including single vesicles, ova vesicles, double and double special vesicles, triple to six vesicles, small and large tubules, vesicles sacs as well as lamellar bodies (Hoog and Lotvall 2015). Based on cell origin, biophysical and biochemical characteristics, biogenesis and release as well as biological functions, EVs are divided into different subtypes (Simon et al. 2018). Three subtypes of EVs are identified including apoptotic bodies (ABs), microvesicles (MVs) and exosomes (EXOs), as shown in figure (1.8) (Simon et al. 2018).

ABs are directly released from the cellular membrane of cells undergoing apoptosis. Their size ranges between 1000-5000 nm in diameter (Lawson et al. 2016). Their cargo contains the fragmented cellular organelles including Golgi apparatus, endoplasmic reticulum or nucleus (Taylor et al. 2008). Furthermore, ABs contribute to horizontal transfer and propagate oncogenes and subsequently increase the tumor formation (Bergsmedh et al. 2001). The voltage-dependent anion channel 1 (VDAC1), which is a mitochondrial protein, could be used as a potential biomarker of ABs (Jeppesen et al. 2014). Regardless of interfering with other vesicles, calreticulin, which is an endoplasmic reticulum protein, could be employed as ABs marker. However, calreticulin was also found to be released with endoplasmic reticulum vesicles namely, microsomes, which are released upon endoplasmic reticulum fragmentation (Jeppesen et al. 2014). Besides these roles, ABs are also known for their immunomodulatory role (Caruso and Poon 2018).
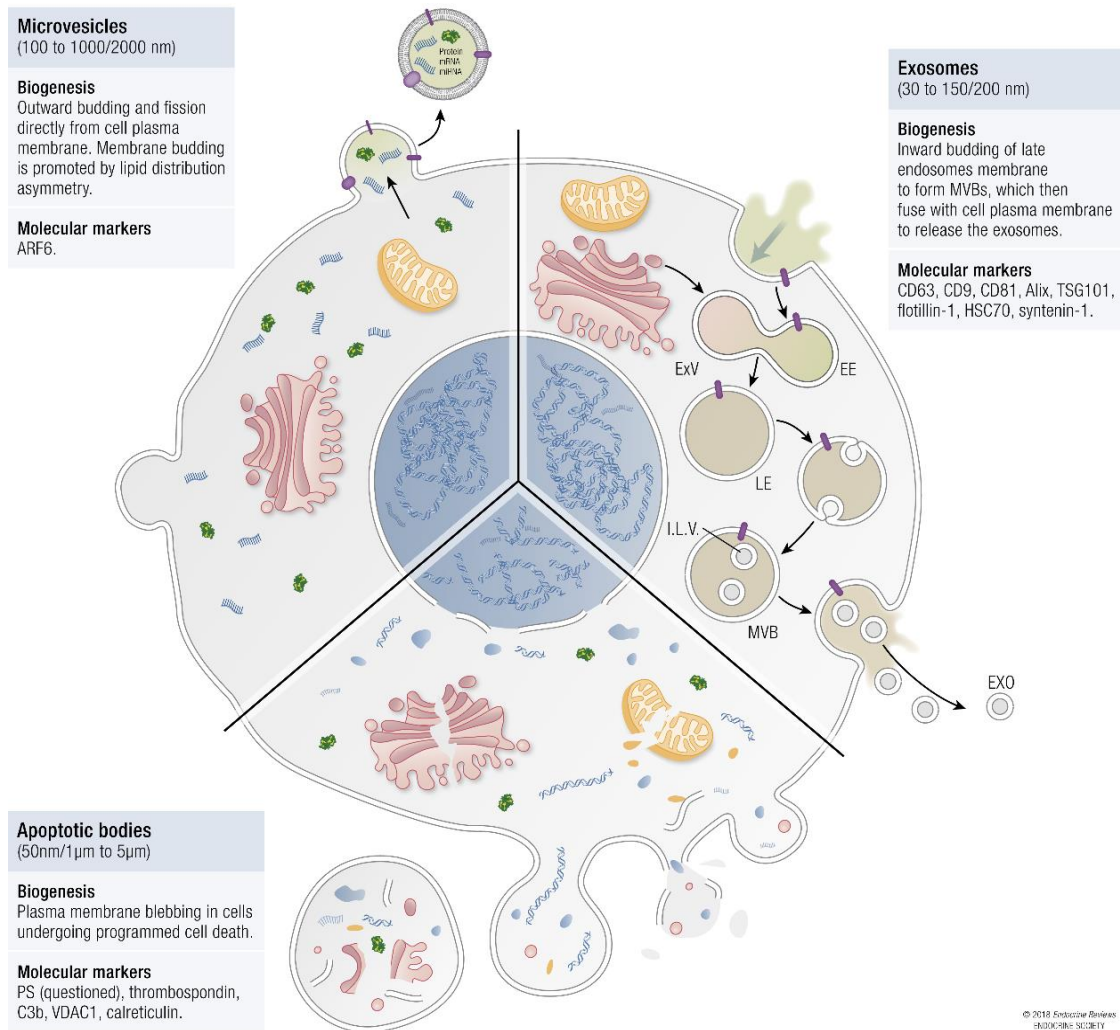
Figure 1.8. Main types of extracellular vesicles (EVs) present in body fluids and culture media, whereby ILVs, intraluminal vesicles; MVBs, multivesicular bodies; EE, early endosome; ExV, exocytic vesicle; LE, late endosome; EXO, exosomes (Simon et al. 2018)

MVs are one of EVs subtype populations, which are characterized by outward budding through direct secretion and fission from the cellular plasma membrane of viable cells. During their release, the blebbing of the cellular plasma membrane is accompanied by membrane proteins remolding and lipids redistribution in order to modulate the rigidity and curvature of the plasma membrane (Tricarico et al. 2017). The MVs size ranges between 50-1000 nm in diameter (El Andaloussi et al. 2013; Thery et al. 2009). Accordingly, they could be sedimented at high-speed centrifugation (31,000 g) (Chargaff and West 1946). They play a vital role in different physiological and pathological functions such as supporting embryo cells (Desrochers et al. 2016) as well as contributing

to embryo-maternal communications (Tong and Chamley 2015). Besides this, their roles were extended to cancer cell progression (Muralidharan-Chari et al. 2010) and invasiveness (Menck et al. 2015).

The term "exosome" was described for the first time in 1981 during the preparation and isolation of MVs from different cell lines (Trams et al. 1981). EXOs are biomolecular nanoparticles released from most of the live cells into the extracellular milieu and unique for each cell origin (de la Torre Gomez et al. 2018). Numerous studies described the size of those vesicles, which is ranged between 20-200 nm in diameter (Lawson et al. 2016). The formation of EXOs occurs intracellularly in the late endosome through invaginations of MVBs limiting membrane. These MVBs-containing EXOs are directly fused to the cellular plasma membrane and subsequently release their contents to extracellular space (Pant et al. 2012). Several potential markers have been identified including tetraspanins (CD63, CD81 and CD9), ALIX, flotillin-1 and TSG101, which are involved in the biogenesis process as shown in Figure 1.9 (Colombo et al. 2014; Jia et al. 2017; Mathivanan et al. 2010). With the growing interest in studying each subtype of vesicle separately, the TSG101 is considered a marker for EXOs (Jeppesen et al. 2014). EXOs have been shown to be implicated in several biological functions such as cellular adaptation (Saeed-Zidane et al. 2017), immune regulation (Muller et al. 2016) as well as tumor maintenance and progression (Suchorska and Lach 2016). Furthermore, they were found to contribute to shuttling the cellular fragmented DNA to the extracellular space in order to maintain cellular homeostasis (Takahashi et al. 2017).

Recently, due to the size overlapping between subtypes, the term of EVs is widely used instead of exosomes or microvesicles (Machtinger et al. 2016; Pavani et al. 2016; Raposo and Stoorvogel 2013). Accordingly, EVs have been coined depending on the origin of cell or tissue. Moreover, several expressions for EVs were used to indicate the source of isolated vesicles such as oviductosomes, uterosomes and prostasomes for oviduct, uterine fluids and seminal fluids, respectively (Machtinger et al. 2016). One of the main obstacles in the EVs experiments is the ability to isolate homogeneous populations with adequate yield from the fluid of interest. Therefore, several isolation methods were developed and adopted to provide the simplest and efficient way of isolation and purification of EVs. These methods were summarized by Simon et al. (2018) with respect to the advantage and limitation of each method (Table 1.2). Following isolation and purification, the characterization of isolated vesicles is considered the cornerstone to discriminate among

subpopulation of EVs. There are different methods and techniques widely used for EVs characterization, namely electron microscopy, nanoparticle tracking analysis, flow cytometry and molecular marker-dependent approach (Pavani et al. 2018; Saeed-Zidane et al. 2017; Simon et al. 2018).
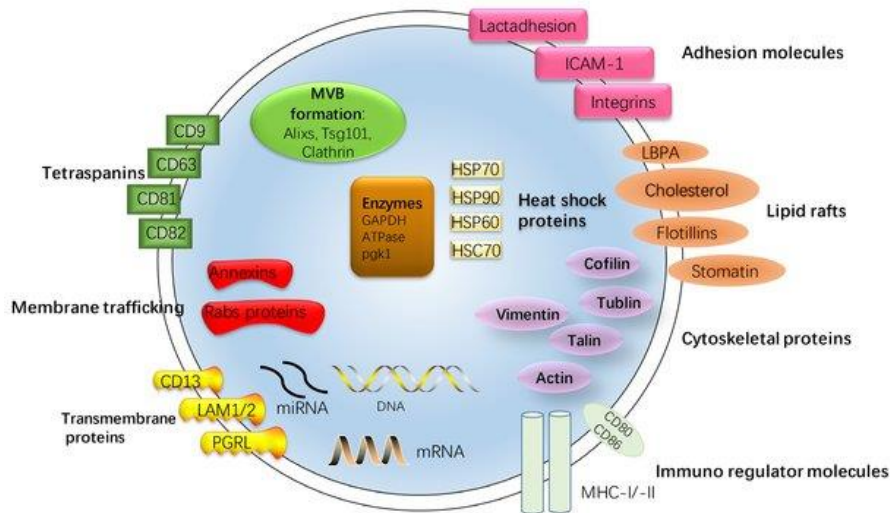
Figure 1.9. Structure and content of exosomes. The lipid bilayer membrane structures of exosomes contain transmembrane proteins and receptors, such as the PGRL, LAMP1/2, CD13, membrane trafficking proteins as annexins and RABs proteins, adhesion molecules as ICAM-1, Lactadhesion and integrins, the lipid raft-associated protein as Flotillins, LBPA, cholesterol, stomatin and tetraspanins, most characteristically CD63 and CD81. Within the exosomal lumen several proteins can be found: cytoskeleton proteins (Vimentin, Cofilin, Tublin, Talin, Actin), heat shock proteins (HSPs), metabolic enzymes (GAPDH, ATPase, pgk1) and protein involving in multivesicular bodies (MVB) formation (AlIX, TSG101, Clathrin). Adopted from Jia et al. (2017)

Table 1.2. Classification of the methods of isolation of EVs based on their principles

Method	Technique	Isolation Principle	General Workflow	Advantage	Limitation
Centrifugation	Serial differential centrifugation	Sedimentation velocity	Serial centrifugation: (1) 300 X *g*, 10 min to remove cells → (2) 2000 X *g*, 10 min to eliminate cell debris and ABs → (3) 10,000-20,000 X *g*, 30 min to isolate MVs → (4) 100,000-200,000 X *g*, 70 min to EXOs segregation	• Popular application • Standardization • Easy to use • Reproducibility • Yield • Affordability over time	• Sedimentation dependent on density, sample viscosity, concentration, and vesicle aggregation
	Density gradient	Buoyant density	Different reagents including sucrose or iodixanol are used during this process. Samples loaded either on top (float down) or at bottom (float up) of gradient and then ultracentrifugation using pre-established criteria.	• Increasing purity of EVs population • EVs homogeneity	• Yield • Reproducibility • Skilled user • Plenty of time
Size exclusion	Filtration	Size/shape	Generally interspersed within centrifugation steps: prior to centrifugation, supernatants are challenged through syringe filters of determined pore size	• Easy to use • More stringency on the populations based on their size • Reproducible • Time-saving	• Loss of yield loss during filtration • Risk of vesicles fragmentation
	Ultrafiltration	Size	Centrifugal filtration units of the prefixed molecular size range that selectively retain vesicles	• Easy to use • Time-saving • Reproducible	• Yield loss within filtering membrane • Risk of vesicles fragmentation
	Chromatography	Size/charge	Purification of EVs based on surface charge or size	• High purity • Lower sensitivity to the viscosity of media • Time-saving	• Centrifugation to remove cell debris • Often issues with volume or buffer associated with elution
Immunoaffinity		Presence of specific EV surface molecules	Microbeads coupled to antibodies are incubated with EVs for specific surface markers recognition (i.e., A33, CD34, CD63). Afterward, beads are washed and recovered by precipitation or magnetism	• Separation based on specific molecules • Selectivity • Resolution • Speed of isolation	• EVs markers are not always known or available • Cost • Yield

Adapted from Simon et al. (2018)

The cell-cell communication role of EVs is evidenced by their transfer of bioactive molecules to target cells such as RNAs (Valadi et al. 2007), DNAs (Takahashi et al. 2017) and proteins (Thery et al. 2002). Besides this, they carry also some metabolites, which are considered as precursors for biological functions, such as sugars, nucleotides, redox regulatory molecules and lipid metabolites in their lumen (Ramirez et al. 2018). Most of the previous studies have focused on the nucleic acids and protein contents. Regarding protein contents, EVs have been shown to be enriched with several proteins including heat shock, plasma membrane, cytoskeletal, cytosolic proteins as well as others involved in EVs trafficking (Yanez-Mo et al. 2015). Moreover, the EVs protein contents could be differed according to cell origin, population of EVs, and isolation methods (Colombo et al. 2014). Different stimulations factors could lead to alteration in the number and contents of EVs (Saeed-Zidane et al. 2017). In addition to protein, lipids are one of the bioactive molecules that could be carried by EVs. The EVs have been revealed to be enriched with certain lipids types, like cholesterol, sphingomyelin (Brouwers et al. 2013; Llorente et al. 2013), ceramide and saturated fatty acids (Trajkovic et al. 2008). However, other lipids were more abundant in donor cells compared to those released via EVs such as phosphatidylglycerols, phosphatidylcholine, and phosphatidylinositols (Llorente et al. 2013).

Valadi and his colleagues reported for the first time that the mast cells released EXOs containing functional miRNAs and RNAs, which could be transferred to recipient cells and alter their physiology (Valadi et al. 2007). With more advanced studies, scientists described the different subtypes of RNAs in EXOs such as mRNA, miRNA, small interfering RNA, ribosomal RNA, transfer RNA, piwi-interacting RNA, and long-noncoding RNA (Huang et al. 2013; van Balkom et al. 2015; Vojtech et al. 2014). Interestingly, the miRNAs profile of endometrial epithelial cells differed from their released EXOs, which indicate their involvement in embryo-maternal communication and explain the selectivity of EVs to their cargo. The bioinformatic studies revealed that the target genes of those miRNAs are involved in embryo implantation (Ng et al. 2013). One of them is miR-30d, which was shown to be actively transmitted to trophoblast cells to enhance embryo implantation (Vilella et al. 2015). Up to date, 289 studies were carried out to identify EVs contents and subsequently promote 9,769 proteins, 3,408 mRNAs, 2,838 miRNAs, and 1,116 lipids entries (http://www.exocarta.org).

1.1.8   EVs biogenesis and uptake

Aforementioned, EXOs biogenesis is initiated within the endosomal system, which is characterized by the presence of MVBs contained vesicles in their lumen formed by inward budding through limiting membrane (Huotari and Helenius 2011; Klumperman and Raposo 2014). Subsequently, MVBs are fused to cellular plasma membrane to release EXOs to extracellular space (de la Torre Gomez et al. 2018; Vlassov et al. 2012), which is facilitated by some membrane proteins including RAB35 and RAB11 resulting in releasing EXOs enriched with flotillin (de la Torre Gomez et al. 2018). During the biogenesis process, the Endosomal Sorting Complex Required for Transport (ESCRT) machinery is believed to play an important role during this process, which was known for their role in the formation and sorting of MVBs in 2001 (Katzmann et al. 2001). Four different protein complexes plus associated proteins including AAA ATPase VPS4 are forming the ESCRT machinery; whereby ESCRT-0 influences the clustering of cargo in a ubiquitin-dependent manner, ESCRT-1 and -2 trigger bud formation, ESCRT-3 boost vesicles separation and the associated proteins permit segregation and recycling of the ESCRT machinery (Hessvik and Llorente 2018; Kowal et al. 2014).

A previous study showed that the depletion of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), a member of ESCRT-0, led to decrease the EXOs secretion in different cell types including HeLa cells (Colombo et al. 2013) and dendritic cells (Tamai et al. 2010). The knockdown of another component of ESCRT-0; namely STEM1 reduced the EXOs secretion in HeLa cells (Colombo et al. 2013). Moreover, the reduction of TSG101, which is a member of ESCRT-1 protein complexes, led also to compromise the number of secreted exosome in Hela cells (Colombo et al. 2013). Additionally, the depletion of TSG101 and CHMP4 resulted in the reduction of released EXOs in MCF-7 cell line (Baietti et al. 2012). However, the depletion of VPS4B was found positively regulate EXOs secretion in HeLa cells (Colombo et al. 2013), while contrary results were obtained from MCF-7 cell line after silencing of two isoforms of VPS4 (Baietti et al. 2012). However, the function of those proteins is not limited to exosome biogenesis, it is extended to other cellular functions like repairing of the cell membrane (Jimenez et al. 2014). Despite depletion of the ESCRT machinery, there is continued formation of MVBs, which may involve tetraspanins, lipids or heat shock protein in this mechanism (Colombo et al. 2013). For instance, impairing ceramide biogenesis via inhibition of neutral sphingomyelinase resulted in a reduction of released

EXOs (Kosaka et al. 2010). Furthermore, the accumulation of cholesterol in the late endosome increased the released EXOs enriched with cholesterol, which had been shown to be flotillin-dependent (Strauss et al. 2010).

The mechanism of mobility and fusion of MVBs with the plasma membrane is controlled by the RAB family. Screening studies showed that inhibition of different members of the RAB family such as RAB27A, RAB27B, RAB35, RAB11 lead to decreasing the released EXOs (Blanc and Vidal 2018; Ostrowski et al. 2010; Takahashi et al. 2017; Yeung et al. 2018). Those proteins have been found to be directly involved in EXOs biogenesis and secretion including transporting of late endosomes to the plasma membrane, regulation of exocytic events, and recycling of membrane components (Blanc and Vidal 2018).

To deliver the content of EVs to their target cells, they initially require to recognize and bind to that cell. There are several mechanisms involved in the internalization of EVs
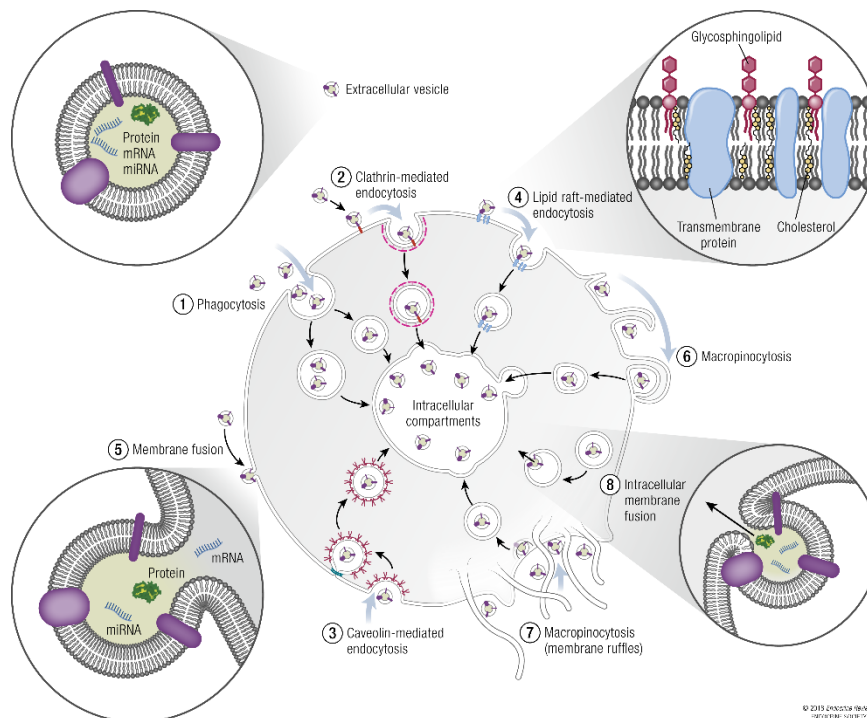
Figure 1.10. Schematic diagram of mechanisms involved in EVs uptake by target cells. EVs have been found to be internalized by target cells through (1) phagocytosis (2) clathrin-mediated endocytosis (3) caveolin-mediated endocytosis (4) lipid rafts (5) fusion with the plasma membrane (6) macropinocytosis (7) membrane ruffles-mediated macropinocytosis (8) fusion with the endosomal limiting membrane following endocytosis (Simon et al. 2018)

contents into target cells as illustrated in Figure 1.10 (Simon et al. 2018). Briefly, they may bind to target cells by direct signaling via ligand or receptors on the surface of recipient cell or by direct fusion of EVs to cellular membrane (Montecalvo et al. 2012), which could be mediated by several mechanisms, like endocytosis, phagocytosis, and micropinocytosis (Escrevente et al. 2011; Feng et al. 2010; Fitzner et al. 2011; Svensson et al. 2013). Adhesion molecules on the surface of EXOs, which mediate the recognition of target cells, could be cell-dependent. For instance, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ were found to be associated with lung cancer, whereas $\alpha_v\beta_4$ associated with liver cancer (Paolillo and Schinelli 2017). Interestingly, the low pH environment is responsible for increasing EXOs tariffing, which could referrer to increase the integrity of EXO membrane (Parolini et al. 2009)

1.1.9   Role of EVs in reproduction

EVs are involved in the most physiological and pathological process including reproduction, such as gametogenesis, fertilization, embryo development, and embryo-maternal interaction (Campoy et al. 2016; Desrochers et al. 2016; Machtinger et al. 2016; Simon et al. 2018; Sohel et al. 2013). The reproduction process is a dynamic system consisting of several stages, where intercellular communication is involved in each stage. EVs have been found in different reproductive tract fluids either in male or female including seminal (Hoog and Lotvall 2015), prostatic (Bryzgunova et al. 2016), follicular (Hung et al. 2015; Hung et al. 2017; Sohel et al. 2013), uterine (Campoy et al. 2016), and oviductal (Qu et al. 2018) fluids. During sperm journey, sperm undergo a series of alteration in morphological and biochemical characteristics, which contribute to acquiring the capability for penetration and fertilization. EVs play a vital role during these processes.

In humans, the released prostasomes, which are EVs obtained from prostate, contain several proteins, which serve as immune regulators, antimicrobials, and antioxidants (Machtinger et al. 2016). Furthermore, they found to contribute to the inhibition of the capacitation process (Pons-Rejraji et al. 2011). In cattle, epididymosomes isolated from different regions of epididymis namely caput, corpus, and cauda differed in their cargo contents (Belleannee et al. 2013; Frenette et al. 2006). The protein contents of this cargo contribute to sperm mobility, fertilization, and protection from oxidative stress injury (Frenette et al. 2006; Frenette et al. 2004). The difference in epididymosomes cargo

among epididymis segments is not limited to proteins but extended to miRNA packed into the cargo (Belleannee et al. 2013).

The maturation of oocyte occurs inside the follicle microenvironment. The follicle contains the oocyte surrounded by cumulus cells followed by mural granulosa, theca cells, and follicular fluids, which is originated from the mixture of circulating blood plasma and products of somatic cells inside the follicle (Machtinger et al. 2016; Revelli et al. 2009). The communication between those cell types and the oocyte is essential for oocyte maturation, growth of the follicle and granulosa cells development, which occurs directly via gap junctions or via different signaling factors including paracrine, endocrine and autocrine.

The discover of EVs in follicular fluid adds an additional way for this bidirectional communication, which was proved either *in vivo* or *in vitro* by uptake of granulosa cells to such vesicles (da Silveira et al. 2012). The content of EVs could differ according to the developmental stage of the follicle (Navakanitworakul et al. 2016) and the female age (da Silveira et al. 2012), which indicate stage-specific function. The miRNAs carried by EVs of small follicles were found to be associated with cell proliferation, whereas those obtained from large follicles were involved in inflammatory response pathways (Navakanitworakul et al. 2016). Moreover, the most abundant EVs miRNA in follicular fluids is predicted to target key genes of different pathways involved in oocyte maturation and follicle growth including transforming growth factor-beta (TGFβ), wingless signaling pathway (WNT), MAPK, EGFR pathway, ubiquitin-mediated pathways, and neurotrophin pathway (da Silveira et al. 2012; Santonocito et al. 2014; Sohel et al. 2013). To elucidate the role of EVs during oocyte maturation, the isolated EVs from follicular fluids of small and large follicle were co-incubated with cumulus-oocyte complexes and the results revealed that the expansion of cumulus cells was increased following co-incubation with isolated EVs from both follicle size with more pronounced effects in small follicle (Hung et al. 2015). It had been shown that the oviductal EXOs are enriched with PMCA4a protein, which is essential for maintaining the homeostasis of sperm Ca^{2+}. This protein was acquired by sperm after *in vitro* co-incubation with oviductosomes (Al-Dossary et al. 2013), which indicate the role of oviductal EVs in maintaining spermatozoa homeostasis and capacitation during their waiting in the oviduct. The CD9-positive EVs are found to be localized on the plasma membrane of oocyte and also on the surface of sperm, which indicates its vital role in sperm-oocyte interaction. In oocyte, the depletion

of CD9 led to a deficiency for fusion with sperm (Runge et al. 2007). Following fertilization, oviductal EVs are involved in enhancing early embryo development. Consequently, the co-culture of *in vitro* oviductal EVs with bovine embryos provoked the development and quality of produced embryos (Lopera-Vasquez et al. 2016). However, the protein profile of released EVs from oviductal cells showed differences in their cargo. The heat shock protein A8 (HSPA8) and oviduct glycoprotein (OVGP), which are significant for fertilization and regulation of early pregnancy, were expressed in *in vivo* EVs, whereas the HSPA8 was not expressed in *in vitro* counterpart (Simon et al. 2018). The implantation process falls on the shoulder of bidirectional coordination between the endometrium and embryo. EVs are known to contribute to this curial part of the pregnancy. Consistently, EVs released from human endometrial epithelial cells contain different miRNA cargo than donor cells. The potential target genes analysis of those miRNA revealed the involvement of those genes in embryo implantation (Ng et al. 2013), which provides a tool for understanding the communication between embryo and endometrial cells during implantation. Moreover, communication among embryonic cells is important for promoting implantation. Triggering the trophoblast migration is found to occur as a result of the attachment of EVs cargo released from inner cell mass contained laminin and fibronectin proteins, which are interacted with integrins on the surface of trophoblast and subsequently activate JNK and FAK signaling cascades. Upon injection of those vesicles into the blastocoel of the blastocysts, the efficiency of implantation was increased (Desrochers et al. 2016). On the other hand, it had been shown that the *in vitro* group culture of embryos was more beneficial for the quality and development of embryo than solitary culture (Kelley and Gardner 2016). This reduction in quality and development in individual cultures embryo was recovered after co-incubation with EVs obtained from grouped culture (Pavani et al. 2018), which indicate the importance of those vesicles during *in vitro* embryo culture in the modulation of embryo-embryo communication in culture microenvironment.

1.2 Hypothesis and objectives

Despite all *in vivo* and/or *in vitro* endeavors to address the potential roles of TFs in cellular oxidative stress defense mechanisms, the role of transcription factors under oxidative stress conditions and their dialog with NRF2 in bovine granulosa cells as well as male and female preimplantation embryos is still unclear.

Therefore, we hypothesized that oxidative stress-mediated transcription factors dysfunction impaired bovine granulosa cell functions and preimplantation embryos, which could be released into extracellular space. Therefore, this dissertation aims to:

1. Investigate the impact of oxidative stress on the expression pattern of transcription factors associated with differentiation and apoptosis as well as their potential cross-talk with NRF2-mediated oxidative stress response in bovine granulosa cells (chapter 2).

2. Investigate the sexually dimorphic expression of transcription factors and their potential release into the extracellular space in bovine preimplantation embryos under oxidative stress (chapter 3).

1.3 Material and methods

To fulfill our aims, three independent experiments were conducted using numerous materials and methods, which are extensively described in the respective sections of this dissertation. Here, the main points of material and methods are briefly described in this chapter.

1.3.1   Bovine granulosa cells culture and H_2O_2 treatment

Bovine ovaries were collected from a local abattoir (Düren, Germany). Granulosa cells were aspirated from small follicles (3-5 mm). Cell viability and concentration were checked using the trypan blue exclusion method. Ultimately, a total of 2.5×10^5 cells per well were cultured in CytoOne® 24-well plate (Starlab GmbH, Hamburg, Germany) containing 600 µL DMEM/F-12 media (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 10% FBS and 1% penicillin/ streptomycin and incubated at 37 °C in 5% CO_2. Thereafter, sub-confluent granulosa cells were treated with 5 µM H_2O_2 for 40 min (Saeed-Zidane et al. 2017) to induce oxidative stress. Twenty-four h post-treatment, cells from all experimental groups throughout the study were subjected to phenotype and genotype analyses.

1.3.2   Small interference RNA transfection

In order to investigate the potential regulation of NRF2 to candidate TFs, small interference RNA (siRNA) technology-mediated NRF2 knockdown was utilized. For this, sub-confluent granulosa cells were transfected with 200 nM siRNA targeting NRF2 (Qiagen, Germany), and then incubated for 24 h. At the end of the incubation period, phenotype assays were performed. Furthermore, the cells were collected and kept at -80 °C for mRNA quantification.

1.3.3   Cell proliferation assay

Granulosa cell proliferation analysis was performed for both control and treated groups of all experiments, 24 h after H_2O_2 challenge or transfection with siRNA targeting NRF2, using CCK-8 kit (Dojindo EU GmbH, München, Germany), according to manufacturer´s protocol. The proliferation rate was indicated by the absorbance at 450 nm wavelength, using Synergy™ H1 Multi-Mode Reader (BioTek Germany, Bad Friedrichshall, Germany).

1.3.4   Mitochondrial activity assay

Granulosa cells were cultured in a slide chamber using the same culture procedure mentioned above and subjected to the candidate treatment. Thereafter, cells were co-incubated with 200 nM MitoTracker red dye (MitoTracker® Red CMXRos, M7512; Invitrogen), for 30 min. Fixed cells were mounted with Vectashield Mounting Medium with DAPI. Finally, the images were acquired using confocal microscopy CLSM LSM-780.

1.3.5   Lipid accumulation assay

Cells cultured in 96-well plate were fixed using 4% paraformaldehyde overnight at 4 °C and then performed to lipid accumulation assay. Briefly, three parts (30 mL) of oil red O (Sigma-Aldrich Chemie GmbH, Munich, Germany) from stock solution with 2 parts (20 mL) deionized water were mixed to prepare the working solution. Cells were co-incubated with 60% isopropanol. After 5 min, isopropanol was poured off and 100 µL of the working solution was pipetted for each well. After the removal of the working solution and washing, images were captured using an inverted microscope with 40X magnification.

1.3.6   DNA fragmentation

The fragmented DNA was isolated according to the Abcam protocol (Cambridge, UK). Briefly, the floated and adhered cells were collected and then lysed using 10 mM Tris (pH 7.4), 5 mM EDTA, 0.2% Triton followed by vortexing. The pellets were pooled together and re-dissolved in 10 mM Tris and 5 mM EDTA. Thereafter, the fragmented DNA was extracted using phenol:chloroform:isoamyl alcohol mixture (25:24:1). Finally, the samples were electrophoretically separated, using 2% agarose gel containing ethidium bromide and then visualized using ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany).

1.3.7   *In vitro* embryo production

The cumulus-oocyte complexes (COCs) were aspirated from small follicles (2-8 mm) of bovine ovaries, using 18 G needle. Oocytes with homogenous cytoplasm and surrounded by multiple layers of compacted cumulus cells were then selected for *in vitro* maturation. Groups of 50 COCs were transferred to modified TCM199 culture media (Sigma Aldrich,

Munich, Germany), supplemented with 55 mg/mL gentamicin and 12% (v/v) heat-inactivated oestrus cow serum (OCS), and then incubated for 22-.24 h at 39 °C under 5% CO_2 in air. Following maturation, the matured oocytes were co-incubated with sex-sorted sperm in F-TALP medium. Afterwards, male and female embryos were transferred to 400 µL of SOF culture medium supplemented with 10% exosomes-depleted fetal bovine serum (FBS) in four-well dishes (Thermo Fisher Scientific, Roskilde; Denmark). Embryos were incubated in two different oxygen levels 5% (physiological level) or 20% (supraphysiological level) until the blastocyst stage (day 8). The spent media was collected and kept at -80 °C for extracellular vesicles isolation.

1.3.8   Intracellular reactive oxygen species (ROS) detection

The level of ROS was detected using the 6-carboxy- 2',7'-dichlorodihydrofluorescein diacetate fluorescent probe (H2DCFDA; Life Technologies, Darmstadt; Germany), following the manufacture's instructions with minimum modifications in terms of concentration. Blastocysts and granulosa cells of all experiment groups throughout the study were co-incubated for 20 min with 5 µM and 75 µM H2DCFDA, respectively. Thereafter, images were immediately acquired under an inverted microscope (Leica DM IRB, Germany), using a green fluorescence filter.

1.3.9   Total cell count of blastocysts

Total cell number was quantified using nuclear fluorescence staining with the glycerol-based Hoechst 33342 (Sigma-Aldrich, Munich, Germany), according to the manufacturer's procedure. For this, blastocysts were fixed in 2% formalin and 0.25% glutaraldehyde for 5 min, and then mounted and stained for 10 min with glycerol-based Hoechst 33342. The stained nuclei were visualized using a fluorescent microscope (Olympus, Tokyo, Japan).

1.3.10 RNA isolation and quantitative Real-Time PCR

Total RNA from granulosa cells and embryos were extracted using miRNeasy® mini kits (Qiagen GmbH, Hilden, Germany) and PicoPure RNA isolation kits (Arcturus, Munich, Germany) following the manufacturer's protocol, respectively. On-column DNA digestion was performed, using RNase-free DNase enzyme (Qiagen GmbH, Hilden, Germany). Additionally, the total RNA from EVs was isolated using Norgen's exosomal

RNA isolation kits following the manufacture's procedure. Quantity and quality of isolated RNAs were assessed using NanoDrop 8000 UV-Vis Spectrophotometers (Thermo Scientific, Wilmington, USA). Equal amounts of total RNA were reverse transcribed using RevertAid first-strand cDNA synthesis kits (Life Technologies GmbH, Darmstadt, Germany). Following cDNA synthesis, the expression level of candidate genes was quantified, using Applied Biosystem® StepOnePlus™ (Applied biosystems, Foster City, CA, USA). The mRNA expression data were analyzed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Melting curve analysis was constructed to verify the specificity of amplification. Following checking the stability of housekeeping genes using NormFinder software, the expression level of ACTB and GAPDH was used for normalization. Additionally, 18S was added to ACTB and GAPDH for normalizing the transcript level of EVs.

1.3.11 Immunoblotting

Total protein was isolated using 1x passive lysis buffer (PLB; Promega GmbH, Mannheim, Germany) and its concentration was determined using Coomassie Protein Assay Reagent (Life Technologies GmbH, Darmstadt, Germany). Thirty-five µg of isolated protein were loaded into SDS-polyacrylamide gel. After electrophoresis, the separated proteins were transferred from SDS-PAGE gel into nitrocellulose membrane (Bio-Rad Laboratories GmbH, Germany), using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories GmbH, Germany). Afterwards, the membrane was blocked in Roti®-Block (Carl Roth GmbH, Germany), for 1 h at room temperature and then incubated overnight at 4 °C with the candidate primary antibodies. Subsequently, the membrane was co-incubated with secondary antibody for 1 h at room temperature Finally, the images were captured, using ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany) followed by stripping with mild stripping buffer and blocking to incubate with another primary antibody.

1.3.12 Protein detection and localization

In situ localization of NRF2 and KLF4 proteins in granulosa cells and embryos was detected, using immunocytochemistry and immunohistochemistry assays, respectively. Samples were fixed overnight, using 4% paraformaldehyde, and then incubated with primary antibody overnight at 4 °C. Afterwards, samples were co-incubated with Alexa

flour goat anti-rabbit secondary antibody. The specimens were mounted on the slide with vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame; California, USA), and visualized under confocal microscopy CLSM LSM-780.

1.3.13 EVs isolation and characterization

The spent culture media from male and female bovine embryos cultured under 5% and 20% oxygen levels were subjected to differential centrifugation, in order to isolate EVs. Finally, the isolated EVs were suspended in PBS-CMF and kept at -80 °C for further investigations. The purity of the isolated EVs was examined, using immunoblotting for the presence of EV marker (CD63) protein and the absence of mitochondrial cellular marker, Cytochrome c (CYCS). Furthermore, the concentration and size of EVs were evaluated using NanoSight NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK), followed by confirmation with electron microscopy.

1.3.14 Statistical analysis

All data were represented as means ± SEM from three and four independent biological replicates obtained from granulosa cells and embryos experiments, respectively. The obtained data were analyzed with GraphPad Prism software version 7 (GraphPad Software, La Jolla, Calif., USA), using different statistical models according to the experimental setup. In granulosa cells experiment, the obtained data were statistically analyzed with student's two-tailed t-test. However, two-way analysis of variance (ANOVA) followed by multiple pair-wise comparisons, using the Tukey post hoc test was used in embryos experiment. The significant difference between means was considered at $P \leq 0.05$.

1.4 Results

Here, the main results of the current dissertation are briefly described below. However, the detailed results are described in the respective chapters.

1.4.1 H_2O_2-induced oxidative stress altered stress-related phenotypes and induced endoplasmic reticulum stress in bovine granulosa cells

Following the exposure of granulosa cells to H_2O_2, the intracellular ROS accumulation and mitochondrial activity were increased and decreased compared to control, which was accompanied by higher DNA fragmentation in the H_2O_2-challenged group. Besides this, the induction of oxidative stress led to increasing the mRNA and protein level of endoplasmic reticulum stress markers.

1.4.2 H_2O_2 challenge altered the mRNA and protein expression patterns of candidate transcription factors in bovine granulosa cells

Upon exposure to oxidative stress conditions, the mRNA and protein levels of NRF2 was significantly induced compared to control ones. This induction was accompanied by a reduction in mRNA level of KLF4 and its protein, followed by alteration of its target genes. Moreover, the mRNA expression level of SREBF1, SREBF2, NOTCH1, c-FOS, KLF6, KLF9 and E2F1 was significantly higher in the H_2O_2-treated group. However, the IRF7 expression level was significantly decreased in H_2O_2-treated granulosa cells.

1.4.3 Disruption in cell apoptosis and differentiation in response to oxidative stress

The mRNA expression level of differentiation marker genes including CYP11A1, STAR and CYP19A1 was significantly upregulated upon exposure of granulosa cells to H_2O_2 challenge. On the other hand, the pro-apoptotic gene (CAPS3) was significantly increased in H_2O_2-challenged cells. In the meanwhile, the anti-apoptotic marker gene (BCL2L1) was noticed to be downregulated, which in turn led to lower cell proliferation rate of granulosa cells cultured under oxidative stress conditions.

1.4.4   Alteration of candidate TFs upon NRF2 knockdown in bovine granulosa cells

The efficiency of transfection was confirmed by showing a reduction in NRF2 transcript and protein levels. Upon NRF2 Knockdown, the mRNA of NOTCH1, KLF9, c-FOS and SREBF2 was significantly downregulated. However, mRNA and protein levels of KLF4 were significantly upregulated accompanied by higher mRNA level of its target genes, especially the BAX gene, and subsequently led to reducing cell proliferation.

1.4.5   Oxidative stress reduced blastocyst rate and induced intracellular ROS accumulation of male and female bovine embryos

Exposure of male and female embryo to higher oxygen level reduced the day 7 and 8 blastocyst rates, which was more pronounced in female embryos. Furthermore, the intracellular ROS accumulation was higher in those embryos cultured under high oxygen level. Moreover, embryos cultured under oxidative stress conditions were found to be lower in their total cell number with a remarkable reduction in female embryos compared to male counterparts.

1.4.6   High oxygen level-induced oxidative stress altered the mRNA and protein expression levels of TFs in male and female embryos

The transcript level of NRF2 was significantly higher in male blastocysts cultured under 20% oxygen level compared to those cultured under 5% oxygen level. However, its protein level was significantly high in both sexes with more abundance in male embryos derived from high oxygen level. Higher expression of NRF2 resulted in higher expression of antioxidant genes (CAT1 and SOD1) in male embryos. Likewise, the mRNA but not protein level of KLF4 was significantly higher in male embryos exposed to higher oxygen tension. However, its target gene, namely POU5F1, was significantly higher in both sexes derived from high oxygen level. On the other hand, NOTCH1 was significantly higher in female embryos cultured under 20% oxygen level compared to male counterparts. Male embryos cultured under both 5% and 20% oxygen levels showed elevated SREBF2 expression pattern compared to female blastocysts cultured under both oxygen levels.

1.4.7   Oxidative stress altered the released EVs and their mRNA contents in male embryos

The data of Nanosight tracking revealed that the concentration of EVs was higher in male embryos cultured under 20% oxygen level compared to those under 5% oxygen level, which was similar to the expression of EVs biogenesis and secretion related genes (ALIX, VPS4B, RAB11FIP1 and RAB27A). Interestingly, EVs released from male embryos cultured under 20% oxygen level was higher in NRF2 mRNA level to other groups. Similarly, SOD1 was significantly higher in EVs released from male embryos exposed to oxidative stress compared to those cultured under normal conditions. The transcript level of CAT1, KLF4 and E2F1 did not show significant differences among all investigated groups.

In conclusion, changing in cellular phenotypes and subsequently functions upon exposure to oxidative stress induced by either hydrogen peroxide (H_2O_2) or oxygen (O_2) level could be attributed to the alteration and cross-talk of TFs in a sex-dependent manner. Additionally, these TFs could be released into extracellular space via EVs, which in turn maintain cellular homeostasis and/or enhance the adaptive response of neighboring cells.

1.5 References

Abuelo A, Hernandez J, Benedito JL, Castillo C (2019) Redox biology in transition periods of dairy cattle: role in the health of periparturient and neonatal animals. Antioxidants (Basel) 8:

Abuelo A, Perez-Santos M, Hernandez J, Castillo C (2014) Effect of colostrum redox balance on the oxidative status of calves during the first 3 months of life and the relationship with passive immune acquisition. Vet J 199:295-299

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S (2012) The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 10:49

Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG (2006) Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 86:503-512

Ahn SG, Thiele DJ (2003) Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. Genes Dev 17:516-528

Aitken RJ, Baker MA (2006) Oxidative stress, sperm survival and fertility control. Mol Cell Endocrinol 250:66-69

Al-Dossary AA, Strehler EE, Martin-Deleon PA (2013) Expression and secretion of plasma membrane Ca2+-ATPase 4a (PMCA4a) during murine estrus: association with oviductal exosomes and uptake in sperm. PLoS One 8:e80181

Alemu TW, Pandey HO, Salilew Wondim D, Gebremedhn S, Neuhof C, Tholen E, Holker M, Schellander K, Tesfaye D (2018) Oxidative and endoplasmic reticulum stress defense mechanisms of bovine granulosa cells exposed to heat stress. Theriogenology 110:130-141

Amin A, Gad A, Salilew-Wondim D, Prastowo S, Held E, Hoelker M, Rings F, Tholen E, Neuhoff C, Looft C, Schellander K, Tesfaye D (2014) Bovine embryo survival under oxidative-stress conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway. Mol Reprod Dev 81:497-513

An T, Qin S, Xu Y, Tang Y, Huang Y, Situ B, Inal JM, Zheng L (2015) Exosomes serve as tumour markers for personalized diagnostics owing to their important role in cancer metastasis. J Extracell Vesicles 4:27522

Anderson HC, Mulhall D, Garimella R (2010) Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. Lab Invest 90:1549-1557

Andoh T, Lee SY, Chiueh CC (2000) Preconditioning regulation of bcl-2 and p66shc by human NOS1 enhances tolerance to oxidative stress. FASEB J 14:2144-2146

Arnér ES (2009) Focus on mammalian thioredoxin reductases--important selenoproteins with versatile functions. Biochim Biophys Acta 1790:495-526

Askoxylaki M, Siristatidis C, Chrelias C, Vogiatzi P, Creatsa M, Salamalekis G, Vrantza T, Vrachnis N, Kassanos D (2013) Reactive oxygen species in the follicular fluid of subfertile women undergoing In Vitro Fertilization: a short narrative review. J Endocrinol Invest 36:1117-1120

Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, Ivarsson Y, Depoortere F, Coomans C, Vermeiren E, Zimmermann P, David G (2012) Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol 14:677-685

Baumgard LH, Collier RJ, Bauman DE (2017) A 100-Year Review: Regulation of nutrient partitioning to support lactation. J Dairy Sci 100:10353-10366

Belleannee C, Calvo E, Caballero J, Sullivan R (2013) Epididymosomes convey different repertoires of microRNAs throughout the bovine epididymis. Biol Reprod 89:30

Ben Mahdi MH, Andrieu V, Pasquier C (2000) Focal adhesion kinase regulation by oxidative stress in different cell types. IUBMB Life 50:291-299

Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, Holmgren L (2001) Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci U S A 98:6407-6411

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2008) Epigenetic differences between male and female bovine blastocysts produced in vitro. Physiol Genomics 32:264-272

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2010) Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proc Natl Acad Sci U S A 107:3394-3399

Bewicke-Copley F, Mulcahy LA, Jacobs LA, Samuel P, Akbar N, Pink RC, Carter DRF (2017) Extracellular vesicles released following heat stress induce bystander effect in unstressed populations. J Extracell Vesicles 6:1340746

Blanc L, Vidal M (2018) New insights into the function of Rab GTPases in the context of exosomal secretion. Small GTPases 9:95-106

Brouwers JF, Aalberts M, Jansen JW, van Niel G, Wauben MH, Stout TA, Helms JB, Stoorvogel W (2013) Distinct lipid compositions of two types of human prostasomes. Proteomics 13:1660-1666

Brown MD, Sacks DB (2009) Protein scaffolds in MAP kinase signalling. Cell Signal 21:462-469

Bryzgunova OE, Zaripov MM, Skvortsova TE, Lekchnov EA, Grigor'eva AE, Zaporozhchenko IA, Morozkin ES, Ryabchikova EI, Yurchenko YB, Voitsitskiy VE, Laktionov PP (2016) Comparative study of extracellular vesicles from the urine of healthy individuals and prostate cancer patients. PLoS One 11:e0157566

Cagnone G, Sirard MA (2016) The embryonic stress response to in vitro culture: insight from genomic analysis. Reproduction 152:R247-R261

Campoy I, Lanau L, Altadill T, Sequeiros T, Cabrera S, Cubo-Abert M, Perez-Benavente A, Garcia A, Borros S, Santamaria A, Ponce J, Matias-Guiu X, Reventos J, Gil-Moreno A, Rigau M, Colas E (2016) Exosome-like vesicles in uterine aspirates: a comparison of ultracentrifugation-based isolation protocols. J Transl Med 14:180

Caro AA, Cederbaum AI (2006) Role of phosphatidylinositol 3-kinase/AKT as a survival pathway against CYP2E1-dependent toxicity. J Pharmacol Exp Ther 318:360-372

Caruso S, Poon IKH (2018) Apoptotic cell-derived extracellular vesicles: more than just debris. Front Immunol 9:1486

Chang L, Kigar SL, Ho JH, Cuarenta A, Gunderson HC, Baldo BA, Bakshi VP, Auger AP (2019) Early life stress alters opioid receptor mRNA levels within the nucleus accumbens in a sex-dependent manner. Brain Res 1710:102-108

Chargaff E, West R (1946) The biological significance of the thromboplastic protein of blood. J Biol Chem 166:189-197

Chen Y, Arsenault R, Napper S, Griebel P (2015) Models and Methods to Investigate Acute Stress Responses in Cattle. Animals (Basel) 5:1268-1295

Chen Y, Li N, Wang J, Zhang X, Pan W, Yu L, Tang B (2019) Enhancement of mitochondrial ROS accumulation and radiotherapeutic efficacy using a Gd-doped titania nanosensitizer. Theranostics 9:167-178

Chiang CY, Chen C (2019) Toward characterizing extracellular vesicles at a single-particle level. J Biomed Sci 26:9

Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, Manel N, Moita LF, Thery C, Raposo G (2013) Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci 126:5553-5565

Colombo M, Raposo G, Thery C (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 30:255-289

Cong X, Zhang Q, Li H, Jiang Z, Cao R, Gao S, Tian W (2017) Puerarin ameliorates heat stress-induced oxidative damage and apoptosis in bovine Sertoli cells by suppressing ROS production and upregulating Hsp72 expression. Theriogenology 88:215-227

da Silveira JC, Veeramachaneni DN, Winger QA, Carnevale EM, Bouma GJ (2012) Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. Biol Reprod 86:71

Dalton TP, Shertzer HG, Puga A (1999) Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol 39:67-101

de la Torre Gomez C, Goreham RV, Bech Serra JJ, Nann T, Kussmann M (2018) "Exosomics"-a review of biophysics, biology and biochemistry of exosomes with a focus on human breast milk. Front Genet 9:92

Desrochers LM, Bordeleau F, Reinhart-King CA, Cerione RA, Antonyak MA (2016) Microvesicles provide a mechanism for intercellular communication by embryonic stem cells during embryo implantation. Nat Commun 7:11958

Di Meo S, Reed TT, Venditti P, Victor VM (2016) Role of ROS and RNS sources in physiological and pathological conditions. Oxid Med Cell Longev 2016:1245049

Diamanti-Kandarakis E, Papalou O, Kandaraki EA, Kassi G (2017) Mechanisms in endocrinology: nutrition as a mediator of oxidative stress in metabolic and reproductive disorders in women. Eur J Endocrinol 176:R79-R99

Ding J, Takano T, Gao S, Han W, Noda C, Yanagi S, Yamamura H (2000) Syk is required for the activation of Akt survival pathway in B cells exposed to oxidative stress. J Biol Chem 275:30873-30877

Ding Q, Keller JN (2001) Proteasome inhibition in oxidative stress neurotoxicity: implications for heat shock proteins. J Neurochem 77:1010-1017

Du B, Takahashi K, Ishida GM, Nakahara K, Saito H, Kurachi H (2006) Usefulness of intraovarian artery pulsatility and resistance indices measurement on the day of follicle aspiration for the assessment of oocyte quality. Fertil Steril 85:366-370

El Andaloussi S, Mager I, Breakefield XO, Wood MJ (2013) Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov 12:347-357

Erickson BH (1966) Development and senescence of the postnatal bovine ovary. J Anim Sci 25:800-805

Escrevente C, Keller S, Altevogt P, Costa J (2011) Interaction and uptake of exosomes by ovarian cancer cells. BMC Cancer 11:108

Farin PW, Farin CE (1995) Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. Biol Reprod 52:676-682

Favetta LA, Robert C, King WA, Betts DH (2004a) Expression profiles of p53 and p66shc during oxidative stress-induced senescence in fetal bovine fibroblasts. Exp Cell Res 299:36-48

Favetta LA, Robert C, St John EJ, Betts DH, King WA (2004b) p66shc, but not p53, is involved in early arrest of in vitro-produced bovine embryos. Mol Hum Reprod 10:383-392

Feng D, Zhao WL, Ye YY, Bai XC, Liu RQ, Chang LF, Zhou Q, Sui SF (2010) Cellular internalization of exosomes occurs through phagocytosis. Traffic 11:675-687

Ferreira de Oliveira JM, Remedios C, Oliveira H, Pinto P, Pinho F, Pinho S, Costa M, Santos C (2014) Sulforaphane induces DNA damage and mitotic abnormalities in human osteosarcoma MG-63 cells: correlation with cell cycle arrest and apoptosis. Nutr Cancer 66:325-334

Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, Regen T, Hanisch UK, Simons M (2011) Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J Cell Sci 124:447-458

Frenette G, Girouard J, Sullivan R (2006) Comparison between epididymosomes collected in the intraluminal compartment of the bovine caput and cauda epididymidis. Biol Reprod 75:885-890

Frenette G, Lessard C, Sullivan R (2004) Polyol pathway along the bovine epididymis. Mol Reprod Dev 69:448-456

Fujii J, Iuchi Y, Okada F (2005) Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. Reprod Biol Endocrinol 3:43

Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D (2012) Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. Biol Reprod 87:100

Gambino V, De Michele G, Venezia O, Migliaccio P, Dall'Olio V, Bernard L, Minardi SP, Della Fazia MA, Bartoli D, Servillo G, Alcalay M, Luzi L, Giorgio M, Scrable H, Pelicci PG, Migliaccio E (2013) Oxidative stress activates a specific p53 transcriptional response that regulates cellular senescence and aging. Aging Cell 12:435-445

Ganeshan L, Li A, O'Neill C (2010) Transformation-related protein 53 expression in the early mouse embryo compromises preimplantation embryonic development by preventing the formation of a proliferating inner cell mass. Biol Reprod 83:958-964

Gao HJ, Zhu YM, He WH, Liu AX, Dong MY, Jin M, Sheng JZ, Huang HF (2012) Endoplasmic reticulum stress induced by oxidative stress in decidual cells: a possible mechanism of early pregnancy loss. Mol Biol Rep 39:9179-9186

Gartel AL, Tyner AL (2002) The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 1:639-649

Girard A, Dufort I, Douville G, Sirard MA (2015) Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. Reprod Biol Endocrinol 13:17

Godet AC, David F, Hantelys F, Tatin F, Lacazette E, Garmy-Susini B, Prats AC (2019) IRES trans-acting factors, key actors of the stress response. Int J Mol Sci 20:

Gómez E, Carrocera S, Martin D, Herrero P, Canela N, Muñoz M (2018) Differential release of cell-signaling metabolites by male and female bovine embryos cultured in vitro. Theriogenology 114:180-184

Gómez E, Gutiérrez-Adán A, Díez C, Bermejo-Alvarez P, Muñoz M, Rodriguez A, Otero J, Alvarez-Viejo M, Martín D, Carrocera S, Caamaño JN (2009) Biological differences between in vitro produced bovine embryos and parthenotes. Reproduction 137:285-295

Goto M, Iwase A, Harata T, Takigawa S, Suzuki K, Manabe S, Kikkawa F (2009) IGF1-induced AKT phosphorylation and cell proliferation are suppressed with the increase in PTEN during luteinization in human granulosa cells. Reproduction 137:835-842

Guo C, Xia Y, Niu P, Jiang L, Duan J, Yu Y, Zhou X, Li Y, Sun Z (2015) Silica nanoparticles induce oxidative stress, inflammation, and endothelial dysfunction in vitro via activation of the MAPK/Nrf2 pathway and nuclear factor-kappaB signaling. Int J Nanomedicine 10:1463-1477

Harding C, Heuser J, Stahl P (1983) Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol 97:329-339

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805-816

Harris SL, Levine AJ (2005) The p53 pathway: positive and negative feedback loops. Oncogene 24:2899-2908

Hessvik NP, Llorente A (2018) Current knowledge on exosome biogenesis and release. Cell Mol Life Sci 75:193-208

Hilali N, Vural M, Camuzcuoglu H, Camuzcuoglu A, Aksoy N (2013) Increased prolidase activity and oxidative stress in PCOS. Clin Endocrinol (Oxf) 79:105-110

Hoog JL, Lotvall J (2015) Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. J Extracell Vesicles 4:28680

Hsueh AJ, Eisenhauer K, Chun SY, Hsu SY, Billig H (1996) Gonadal cell apoptosis. Recent Prog Horm Res 51:433-455; discussion 455-436

Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, Liang M, Dittmar RL, Liu Y, Liang M, Kohli M, Thibodeau SN, Boardman L, Wang L (2013) Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC Genomics 14:319

Hung WT, Hong X, Christenson LK, McGinnis LK (2015) Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biol Reprod 93:117

Hung WT, Navakanitworakul R, Khan T, Zhang P, Davis JS, McGinnis LK, Christenson LK (2017) Stage-specific follicular extracellular vesicle uptake and regulation of bovine granulosa cell proliferation. Biol Reprod 97:644-655

Huotari J, Helenius A (2011) Endosome maturation. EMBO J 30:3481-3500

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76-86

Jeppesen DK, Hvam ML, Primdahl-Bengtson B, Boysen AT, Whitehead B, Dyrskjot L, Orntoft TF, Howard KA, Ostenfeld MS (2014) Comparative analysis of discrete exosome fractions obtained by differential centrifugation. J Extracell Vesicles 3:25011

Jia Y, Chen Y, Wang Q, Jayasinghe U, Luo X, Wei Q, Wang J, Xiong H, Chen C, Xu B, Hu W, Wang L, Zhao W, Zhou J (2017) Exosome: emerging biomarker in breast cancer. Oncotarget 8:41717-41733

Jimenez AJ, Maiuri P, Lafaurie-Janvore J, Divoux S, Piel M, Perez F (2014) ESCRT machinery is required for plasma membrane repair. Science 343:1247136

Kadenbach B, Arnold S, Lee I, Huttemann M (2004) The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. Biochim Biophys Acta 1655:400-408

Kala M, Shaikh MV, Nivsarkar M (2017) Equilibrium between anti-oxidants and reactive oxygen species: a requisite for oocyte development and maturation. Reprod Med Biol 16:28-35

Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M (2005) Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120:649-661

Katzmann DJ, Babst M, Emr SD (2001) Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell 106:145-155

Kelley RL, Gardner DK (2016) Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. Reprod Biomed Online 33:537-549

Kikuchi H, Kuribayashi F, Takami Y, Imajoh-Ohmi S, Nakayama T (2011) GCN5 regulates the activation of PI3K/Akt survival pathway in B cells exposed to oxidative stress via controlling gene expressions of Syk and Btk. Biochem Biophys Res Commun 405:657-661

Kim AH, Khursigara G, Sun X, Franke TF, Chao MV (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol 21:893-901

Kim EK, Choi EJ (2010) Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802:396-405

Klumperman J, Raposo G (2014) The complex ultrastructure of the endolysosomal system. Cold Spring Harb Perspect Biol 6:a016857

Kobayashi S, Isotani A, Mise N, Yamamoto M, Fujihara Y, Kaseda K, Nakanishi T, Ikawa M, Hamada H, Abe K, Okabe M (2006) Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. Curr Biol 16:166-172

Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T (2010) Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 285:17442-17452

Kowal J, Tkach M, Thery C (2014) Biogenesis and secretion of exosomes. Curr Opin Cell Biol 29:116-125

Kruse JP, Gu W (2009) Modes of p53 regulation. Cell 137:609-622

Kumar A, Mandal A, Gupta AK, Ratwan P (2016) Genetic and environmental causes of variation in gestation length of Jersey crossbred cattle. Vet World 9:351-355

Kumar B, Manuja A, Aich P (2012) Stress and its impact on farm animals. Front Biosci (Elite Ed) 4:1759-1767

Lasser C, Alikhani VS, Ekstrom K, Eldh M, Paredes PT, Bossios A, Sjostrand M, Gabrielsson S, Lotvall J, Valadi H (2011) Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Transl Med 9:9

Lawson C, Vicencio JM, Yellon DM, Davidson SM (2016) Microvesicles and exosomes: new players in metabolic and cardiovascular disease. J Endocrinol 228:R57-71

Lee MN, Lee SH, Lee MY, Kim YH, Park JH, Ryu JM, Yun SP, Lee YJ, Kim MO, Park K, Han HJ (2008) Effect of dihydrotestosterone on mouse embryonic stem cells exposed to H2O2-induced oxidative stress. J Vet Sci 9:247-256

Lee SW, Fang L, Igarashi M, Ouchi T, Lu KP, Aaronson SA (2000) Sustained activation of Ras/Raf/mitogen-activated protein kinase cascade by the tumor suppressor p53. Proc Natl Acad Sci U S A 97:8302-8305

Leite RF, Annes K, Ispada J, de Lima CB, Dos Santos EC, Fontes PK, Nogueira MFG, Milazzotto MP (2017) Oxidative stress alters the profile of transcription factors related to early development on in vitro produced embryos. Oxid Med Cell Longev 2017:1502489

Li L, Tan H, Yang H, Li F, He X, Gu Z, Zhao M, Su L (2017) Reactive oxygen species mediate heat stress-induced apoptosis via ERK dephosphorylation and Bcl-2 ubiquitination in human umbilical vein endothelial cells. Oncotarget 8:12902-12916

Li N, Alam J, Venkatesan MI, Eiguren-Fernandez A, Schmitz D, Di Stefano E, Slaughter N, Killeen E, Wang X, Huang A, Wang M, Miguel AH, Cho A, Sioutas C, Nel AE (2004) Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. J Immunol 173:3467-3481

Lima MH, Souza LC, Caperuto LC, Bevilacqua E, Gasparetti AL, Zanuto R, Saad MJ, Carvalho CR (2006) Up-regulation of the phosphatidylinositol 3-kinase/protein kinase B pathway in the ovary of rats by chronic treatment with hCG and insulin. J Endocrinol 190:451-459

Llorente A, Skotland T, Sylvanne T, Kauhanen D, Rog T, Orlowski A, Vattulainen I, Ekroos K, Sandvig K (2013) Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. Biochim Biophys Acta 1831:1302-1309

Lopera-Vasquez R, Hamdi M, Fernandez-Fuertes B, Maillo V, Beltran-Brena P, Calle A, Redruello A, Lopez-Martin S, Gutierrez-Adan A, Yanez-Mo M, Ramirez MA, Rizos D (2016) Extracellular vesicles from BOEC in in vitro embryo development and quality. PLoS One 11:e0148083

Lowe R, Gemma C, Rakyan VK, Holland ML (2015) Sexually dimorphic gene expression emerges with embryonic genome activation and is dynamic throughout development. BMC Genomics 16:295

Lu J, Wang Z, Cao J, Chen Y, Dong Y (2018) A novel and compact review on the role of oxidative stress in female reproduction. Reprod Biol Endocrinol 16:80

Machtinger R, Laurent LC, Baccarelli AA (2016) Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. Hum Reprod Update 22:182-193

Mahadev K, Zilbering A, Zhu L, Goldstein BJ (2001) Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. J Biol Chem 276:21938-21942

Marinho HS, Real C, Cyrne L, Soares H, Antunes F (2014) Hydrogen peroxide sensing, signaling and regulation of transcription factors. Redox Biol 2:535-562

Martindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192:1-15

Mathevon M, Buhr MM, Dekkers JC (1998) Environmental, management, and genetic factors affecting semen production in Holstein bulls. J Dairy Sci 81:3321-3330

Mathivanan S, Ji H, Simpson RJ (2010) Exosomes: extracellular organelles important in intercellular communication. J Proteomics 73:1907-1920

Menck K, Scharf C, Bleckmann A, Dyck L, Rost U, Wenzel D, Dhople VM, Siam L, Pukrop T, Binder C, Klemm F (2015) Tumor-derived microvesicles mediate human breast cancer invasion through differentially glycosylated EMMPRIN. J Mol Cell Biol 7:143-153

Meng D, Shi X, Jiang BH, Fang J (2007) Insulin-like growth factor-I (IGF-I) induces epidermal growth factor receptor transactivation and cell proliferation through reactive oxygen species. Free Radic Biol Med 42:1651-1660

Meves A, Stock SN, Beyerle A, Pittelkow MR, Peus D (2001) H(2)O(2) mediates oxidative stress-induced epidermal growth factor receptor phosphorylation. Toxicol Lett 122:205-214

Mo JS, Yoon JH, Ann EJ, Ahn JS, Baek HJ, Lee HJ, Kim SH, Kim YD, Kim MY, Park HS (2013) Notch1 modulates oxidative stress induced cell death through suppression of apoptosis signal-regulating kinase 1. Proc Natl Acad Sci U S A 110:6865-6870

Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE (2012) Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119:756-766

Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol Cell Biol 20:7146-7159

Muller L, Mitsuhashi M, Simms P, Gooding WE, Whiteside TL (2016) Tumor-derived exosomes regulate expression of immune function-related genes in human T cell subsets. Sci Rep 6:20254

Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C (2010) Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci 123:1603-1611

Murdoch WJ (1998) Inhibition by oestradiol of oxidative stress-induced apoptosis in pig ovarian tissues. J Reprod Fertil 114:127-130

Myatt L, Cui X (2004) Oxidative stress in the placenta. Histochem Cell Biol 122:369-382

Nagai H, Noguchi T, Takeda K, Ichijo H (2007) Pathophysiological roles of ASK1-MAP kinase signaling pathways. J Biochem Mol Biol 40:1-6

Namani A, Li Y, Wang XJ, Tang X (2014) Modulation of NRF2 signaling pathway by nuclear receptors: implications for cancer. Biochim Biophys Acta 1843:1875-1885

Navakanitworakul R, Hung WT, Gunewardena S, Davis JS, Chotigeat W, Christenson LK (2016) Characterization and small RNA content of extracellular vesicles in follicular fluid of developing bovine antral follicles. Sci Rep 6:25486

Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL, Salamonsen LA (2013) Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. PLoS One 8:e58502

Nguyen T, Nioi P, Pickett CB (2009) The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 284:13291-13295

Nishihara T, Matsumoto K, Hosoi Y, Morimoto Y (2018) Evaluation of antioxidant status and oxidative stress markers in follicular fluid for human in vitro fertilization outcome. Reprod Med Biol 17:481-486

Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, Moita CF, Schauer K, Hume AN, Freitas RP, Goud B, Benaroch P, Hacohen N, Fukuda M, Desnos C, Seabra MC, Darchen F, Amigorena S, Moita LF, Thery C (2010) Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol 12:19-30; sup pp 11-13

Pant S, Hilton H, Burczynski ME (2012) The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. Biochem Pharmacol 83:1484-1494

Paolillo M, Schinelli S (2017) Integrins and exosomes, a dangerous liaison in cancer progression. Cancers (Basel) 9:

Park HS, Lee JS, Huh SH, Seo JS, Choi EJ (2001) Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. EMBO J 20:446-456

Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, Coscia C, Iessi E, Logozzi M, Molinari A, Colone M, Tatti M, Sargiacomo M, Fais S (2009) Microenvironmental pH is a key factor for exosome traffic in tumor cells. J Biol Chem 284:34211-34222

Pavani KC, Alminana C, Wydooghe E, Catteeuw M, Ramirez MA, Mermillod P, Rizos D, Van Soom A (2016) Emerging role of extracellular vesicles in communication of preimplantation embryos in vitro. Reprod Fertil Dev 29:66-83

Pavani KC, Hendrix A, Van Den Broeck W, Couck L, Szymanska K, Lin X, De Koster J, Van Soom A, Leemans B (2018) Isolation and characterization of functionally active extracellular vesicles from culture medium conditioned by bovine embryos in vitro. Int J Mol Sci 20:

Perbal B (2003) Communication is the key. Cell Commun Signal 1:3

Pérez-Cerezales S, Ramos-Ibeas P, Rizos D, Lonergan P, Bermejo-Alvarez P, Gutierrez-Adan A (2018) Early sex-dependent differences in response to environmental stress. Reproduction 155:R39-R51

Pons-Rejraji H, Artonne C, Sion B, Brugnon F, Canis M, Janny L, Grizard G (2011) Prostasomes: inhibitors of capacitation and modulators of cellular signalling in human sperm. Int J Androl 34:568-580

Pontes JH, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, Barreiros TR, Oliveira JA, Hasler JF, Seneda MM (2009) Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. Theriogenology 71:690-697

Qi H, Han Y, Rong J (2012) Potential roles of PI3K/Akt and Nrf2-Keap1 pathways in regulating hormesis of Z-ligustilide in PC12 cells against oxygen and glucose deprivation. Neuropharmacology 62:1659-1670

Qu P, Zhao Y, Wang R, Zhang Y, Li L, Fan J, Liu E (2018) Extracellular vesicles derived from donor oviduct fluid improved birth rates after embryo transfer in mice. Reprod Fertil Dev

Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, Dhama K (2014) Oxidative stress, prooxidants, and antioxidants: the interplay. Biomed Res Int 2014:761264

Ramirez MI, Amorim MG, Gadelha C, Milic I, Welsh JA, Freitas VM, Nawaz M, Akbar N, Couch Y, Makin L, Cooke F, Vettore AL, Batista PX, Freezor R, Pezuk JA, Rosa-Fernandes L, Carreira ACO, Devitt A, Jacobs L, Silva IT, Coakley G, Nunes DN, Carter D, Palmisano G, Dias-Neto E (2018) Technical challenges of working with extracellular vesicles. Nanoscale 10:881-906

Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 200:373-383

Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P (2009) Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol 7:40

Roth Z (2017) Effect of heat stress on reproduction in dairy cows: Insights into the cellular and molecular responses of the oocyte. Annu Rev Anim Biosci 5:151-170

Runge KE, Evans JE, He ZY, Gupta S, McDonald KL, Stahlberg H, Primakoff P, Myles DG (2007) Oocyte CD9 is enriched on the microvillar membrane and required for normal microvillar shape and distribution. Dev Biol 304:317-325

Saeed-Zidane M, Linden L, Salilew-Wondim D, Held E, Neuhoff C, Tholen E, Hoelker M, Schellander K, Tesfaye D (2017) Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress. PLoS One 12:e0187569

Salhab M, Tosca L, Cabau C, Papillier P, Perreau C, Dupont J, Mermillod P, Uzbekova S (2011) Kinetics of gene expression and signaling in bovine cumulus cells throughout IVM in different mediums in relation to oocyte developmental competence, cumulus apoptosis and progesterone secretion. Theriogenology 75:90-104

Salilew-Wondim D, Fournier E, Hoelker M, Saeed-Zidane M, Tholen E, Looft C, Neuhoff C, Besenfelder U, Havlicek V, Rings F, Gagne D, Sirard MA, Robert C, Shojaei Saadi HA, Gad A, Schellander K, Tesfaye D (2015) Genome-wide DNA methylation patterns of bovine blastocysts developed in vivo from embryos completed different stages of development in vitro. PLoS One 10:e0140467

Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, Barbagallo D, Borzi P, Rizzari S, Maugeri M, Scollo P, Tatone C, Valadi H, Purrello M, Di Pietro C (2014) Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. Fertil Steril 102:1751-1761 e1751

Scandalios JG (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res 38:995-1014

Shi X, Zhou B (2010) The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. Toxicol Sci 115:391-400

Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N (2011) Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci U S A 108:1462-1467

Simon C, Greening DW, Bolumar D, Balaguer N, Salamonsen LA, Vilella F (2018) Extracellular vesicles in human reproduction in health and disease. Endocr Rev 39:292-332

Sohel MM, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K, Tesfaye D (2013) Exosomal and non-exosomal transport of extra-cellular microRNAs in follicular fluid: implications for bovine oocyte developmental competence. PLoS One 8:e78505

Sohel MMH, Akyuz B, Konca Y, Arslan K, Sariozkan S, Cinar MU (2019) Oxidative stress modulates the expression of apoptosis-associated microRNAs in bovine granulosa cells in vitro. Cell Tissue Res

Son Y, Cheong YK, Kim NH, Chung HT, Kang DG, Pae HO (2011) Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? J Signal Transduct 2011:792639

Sonoda Y, Watanabe S, Matsumoto Y, Aizu-Yokota E, Kasahara T (1999) FAK is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of a human glioblastoma cell line. J Biol Chem 274:10566-10570

Sordillo LM, Aitken SL (2009) Impact of oxidative stress on the health and immune function of dairy cattle. Vet Immunol Immunopathol 128:104-109

Strauss K, Goebel C, Runz H, Mobius W, Weiss S, Feussner I, Simons M, Schneider A (2010) Exosome secretion ameliorates lysosomal storage of cholesterol in Niemann-Pick type C disease. J Biol Chem 285:26279-26288

Suchorska WM, Lach MS (2016) The role of exosomes in tumor progression and metastasis (Review). Oncol Rep 35:1237-1244

Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, Taketo MM, Yamamoto M, Igarashi K (2002) Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. EMBO J 21:5216-5224

Sundrani DP, Roy SS, Jadhav AT, Joshi SR (2017) Sex-specific differences and developmental programming for diseases in later life. Reprod Fertil Dev 29:2085-2099

Svensson KJ, Christianson HC, Wittrup A, Bourseau-Guilmain E, Lindqvist E, Svensson LM, Morgelin M, Belting M (2013) Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. J Biol Chem 288:17713-17724

Takahashi A, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S, Takasugi M, Watanabe S, Kanemaki MT, Obuse C, Hara E (2017) Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. Nat Commun 8:15287

Takano T, Sada K, Yamamura H (2002) Role of protein-tyrosine kinase syk in oxidative stress signaling in B cells. Antioxid Redox Signal 4:533-541

Tamai K, Tanaka N, Nakano T, Kakazu E, Kondo Y, Inoue J, Shiina M, Fukushima K, Hoshino T, Sano K, Ueno Y, Shimosegawa T, Sugamura K (2010) Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. Biochem Biophys Res Commun 399:384-390

Tan M, Li S, Swaroop M, Guan K, Oberley LW, Sun Y (1999) Transcriptional activation of the human glutathione peroxidase promoter by p53. J Biol Chem 274:12061-12066

Taylor RC, Cullen SP, Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 9:231-241

Tetreault MP, Yang Y, Katz JP (2013) Krüppel-like factors in cancer. Nat Rev Cancer 13:701-713

Thery C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9:581-593

Thery C, Zitvogel L, Amigorena S (2002) Exosomes: composition, biogenesis and function. Nat Rev Immunol 2:569-579

Tiberi L, Faisal A, Rossi M, Di Tella L, Franceschi C, Salvioli S (2006) p66(Shc) gene has a pro-apoptotic role in human cell lines and it is activated by a p53-independent pathway. Biochem Biophys Res Commun 342:503-508

Tong M, Chamley LW (2015) Placental extracellular vesicles and feto-maternal communication. Cold Spring Harb Perspect Med 5:a023028

Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brugger B, Simons M (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 319:1244-1247

Trams EG, Lauter CJ, Salem N, Jr., Heine U (1981) Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim Biophys Acta 645:63-70

Tricarico C, Clancy J, D'Souza-Schorey C (2017) Biology and biogenesis of shed microvesicles. Small GTPases 8:220-232

Tsunoda S, Kimura N, Fujii J (2014) Oxidative stress and redox regulation of gametogenesis, fertilization, and embryonic development. Reprod Med Biol 13:71-79

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654-659

van Balkom BW, Eisele AS, Pegtel DM, Bervoets S, Verhaar MC (2015) Quantitative and qualitative analysis of small RNAs in human endothelial cells and exosomes provides insights into localized RNA processing, degradation and sorting. J Extracell Vesicles 4:26760

Vandaele L, Thys M, Bijttebier J, Van Langendonckt A, Donnay I, Maes D, Meyer E, Van Soom A (2010) Short-term exposure to hydrogen peroxide during oocyte maturation improves bovine embryo development. Reproduction 139:505-511

Vanselow J, Vernunft A, Koczan D, Spitschak M, Kuhla B (2016) Exposure of Lactating Dairy Cows to Acute Pre-Ovulatory Heat Stress Affects Granulosa Cell-Specific Gene Expression Profiles in Dominant Follicles. PLoS One 11:e0160600

Vilella F, Moreno-Moya JM, Balaguer N, Grasso A, Herrero M, Martinez S, Marcilla A, Simon C (2015) Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. Development 142:3210-3221

Vlassov AV, Magdaleno S, Setterquist R, Conrad R (2012) Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochim Biophys Acta 1820:940-948

Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud RP, Strobl J, Westerberg K, Gottardo R, Tewari M, Hladik F (2014) Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. Nucleic Acids Res 42:7290-7304

Volloch V, Gabai VL, Rits S, Force T, Sherman MY (2000) HSP72 can protect cells from heat-induced apoptosis by accelerating the inactivation of stress kinase JNK. Cell Stress Chaperones 5:139-147

Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, Imakado S, Kotsuji T, Otsuka F, Roop DR, Harada T, Engel JD, Yamamoto M (2003) Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. Nat Genet 35:238-245

Walsh SW, Williams EJ, Evans AC (2011) A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci 123:127-138

Wang C, Li X, Wang H, Xie Q, Xu Y (2014) Notch1-nuclear factor kappaB involves in oxidative stress-induced alcoholic steatohepatitis. Alcohol Alcohol 49:10-16

Wang L, Xu T, Lei WW, Liu DM, Li YJ, Xuan RJ, Ma JJ (2011) Cadmium-induced oxidative stress and apoptotic changes in the testis of freshwater crab, Sinopotamon henanense. PLoS One 6:e27853

Wang S, He G, Chen M, Zuo T, Xu W, Liu X (2017) The Role of Antioxidant Enzymes in the Ovaries. Oxid Med Cell Longev 2017:4371714

Wang X, McCullough KD, Franke TF, Holbrook NJ (2000) Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. J Biol Chem 275:14624-14631

Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ, Jr., Harris CC (1999) GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci U S A 96:3706-3711

Weng Q, Liu Z, Li B, Liu K, Wu W, Liu H (2016) Oxidative stress induces mouse follicular granulosa cells apoptosis via JNK/FoxO1 pathway. PLoS One 11:e0167869

Willi J, Kupfer P, Evequoz D, Fernandez G, Katz A, Leumann C, Polacek N (2018) Oxidative stress damages rRNA inside the ribosome and differentially affects the catalytic center. Nucleic Acids Res 46:1945-1957

Wisdom SJ, Wilson R, McKillop JH, Walker JJ (1991) Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. Am J Obstet Gynecol 165:1701-1704

Wolf P (1967) The nature and significance of platelet products in human plasma. Br J Haematol 13:269-288

Woo HA, Yim SH, Shin DH, Kang D, Yu DY, Rhee SG (2010) Inactivation of peroxiredoxin I by phosphorylation allows localized H_2O_2 accumulation for cell signaling. Cell 140:517-528

Xu X, Li H, Hou X, Li D, He S, Wan C, Yin P, Liu M, Liu F, Xu J (2015) Punicalagin induces Nrf2/HO-1 expression via upregulation of PI3K/AKT pathway and inhibits LPS-induced oxidative stress in RAW264.7 macrophages. Mediators Inflamm 2015:380218

Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borras FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colas E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Kramer-Albers EM, Laitinen S, Lasser C, Lener T, Ligeti E, Line A, Lipps G, Llorente A, Lotvall J, Mancek-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen EN, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pallinger E, Del Portillo HA, Reventos J, Rigau M, Rohde E, Sammar M, Sanchez-Madrid F, Santarem N, Schallmoser K, Ostenfeld MS, Stoorvogel W, Stukelj R, Van der Grein SG, Vasconcelos MH, Wauben MH, De Wever O (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4:27066

Yang H, Xie Y, Yang D, Ren D (2017) Oxidative stress-induced apoptosis in granulosa cells involves JNK, p53 and Puma. Oncotarget 8:25310-25322

Yeung V, Webber JP, Dunlop EA, Morgan H, Hutton J, Gurney M, Jones E, Falcon-Perez J, Tabi Z, Errington R, Clayton A (2018) Rab35-dependent extracellular nanovesicles are required for induction of tumour supporting stroma. Nanoscale 10:8547-8559

Yin Y, Terauchi Y, Solomon GG, Aizawa S, Rangarajan PN, Yazaki Y, Kadowaki T, Barrett JC (1998) Involvement of p85 in p53-dependent apoptotic response to oxidative stress. Nature 391:707-710

Zabet NR, Adryan B (2013) The effects of transcription factor competition on gene regulation. Front Genet 4:197

Zhang JQ, Gao BW, Wang J, Ren QL, Chen JF, Ma Q, Zhang ZJ, Xing BS (2016) Critical role of FoxO1 in granulosa cell apoptosis caused by oxidative stress and protective effects of grape seed procyanidin B2. Oxid Med Cell Longev 2016:6147345

Zhou JY, Liu Y, Wu GS (2006) The role of mitogen-activated protein kinase phosphatase-1 in oxidative damage-induced cell death. Cancer Res 66:4888-4894

Zucker SN, Fink EE, Bagati A, Mannava S, Bianchi-Smiraglia A, Bogner PN, Wawrzyniak JA, Foley C, Leonova KI, Grimm MJ, Moparthy K, Ionov Y, Wang J, Liu S, Sexton S, Kandel ES, Bakin AV, Zhang Y, Kaminski N, Segal BH, Nikiforov MA (2014) Nrf2 amplifies oxidative stress via induction of Klf9. Mol Cell 53:916-928

Chapter 2: The role and regulation of transcription factors under oxidative stress conditions in bovine granulosa cells

(Manuscript under preparation, to be submitted to Cell and Tissue Research)

Differential regulation of transcription factors is associated with bovine granulosa cell functions under oxidative stress condition

Mohamed Omar Taqi[1], Mohammed Saeed-Zidane[1], Samuel Gebremedhn[1], Dessie Salilew-Wondim[1], Ernst Tholen[1], Christiane Neuhoff[1], Michael Hoelker[1,2,3], Karl Schellander[1,3], Dawit Tesfaye[1,3*]

[1]Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn
[2]Teaching and Research Station Frankenforst, University of Bonn, Königswinter, Germany
[3]Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

[*]Correspondence to:
Institute of Animal Science
Animal Breeding and Husbandry Group
Endenicher Allee 15,
Bonn 53115, Germany
E-mail: tesfaye@itw.uni-bonn.de

2.1 Abstract

Transcription factors (TFs) are known to be involved in mammalian folliculogenesis. However, the regulatory role of TFs during oxidative stress is not fully understood. Hence, the current study was aimed to investigate the regulatory role of the TFs during the development of bovine granulosa cells (bGCs) under oxidative stress (OS) conditions. For this, sub-confluent bGCs were treated with H_2O_2 to induce OS. Twenty-four h later, cells were subjected to various phenotypic and gene expression analysis including TFs, endoplasmic reticulum stress, apoptosis, cell proliferation and differentiation markers. In response to OS, bGCs exhibited higher reactive oxygen species accumulation, DNA fragmentation and endoplasmic reticulum stress accompanied by reduction of mitochondrial activity. In addition, higher lipid accumulation and lower cell proliferation were noticed in H_2O_2-challenged cells. The mRNA level of TFs including NRF2, E2F1, KLF6, KLF9, c-FOS, SREBF1, SREBF2 and NOTCH1 was increased in H_2O_2-treated cells compared to control counterparts. However, the expression level of KLF4 and its downstream gene, CCNB1, were decreased in H_2O_2-challenged group. Moreover, targeted inhibition of NRF2 using small interference RNA resulted in reduced expression of KLF9, c-FOS, SREBF2 and NOTCH1 genes, while the expression of KLF4 was upregulated. Taken together, bovine granulosa cells exposed to oxidative stress exhibited differential expression of various transcription factors which are mediated by the NRF2 signaling pathway.

Keywords: Transcription factors, Granulosa cells, Oxidative stress, Apoptosis and differentiation, NRF2 cross-talk

2.2 Introduction

During folliculogenesis, the oocyte is surrounded by multiple layers of somatic cells including granulosa cells, which significantly contribute to the production of hormones including estradiol and progesterone (Wen et al. 2010). Thus, disruption of granulosa cell functions results in poor oocyte quality and subsequently reduce the pregnancy rate (Lai et al. 2018). Indeed, the majority of the growing follicles undergo follicular atresia (Roche and Boland 1991; Shen et al. 2012). Previous evidence has been reported that exposure of granulosa cells to oxidative stress led to impair their functions and subsequently poor quantity and quality oocytes (Jancar et al. 2007; Lai et al. 2018; Rajani et al. 2012). On the other hand, the follicular fluid of the atretic and preovulatory follicles is characterized by its enrichment in reactive oxygen species (ROS) (Hennet et al. 2013). This was further supported by that elevated ROS levels at the time of ovulation play an indispensable role in facilitating ovulation process (Tanabe et al. 2015), which could be inhibited by antioxidants administration (Shkolnik et al. 2011).

ROS are endogenously produced during metabolic pathways as a byproduct from different cellular compartments including the plasma membrane, mitochondria, endoplasmic reticulum and peroxisome (Di Meo et al. 2016). Cells are equipped with a well-balanced system including antioxidants machinery to eliminate the excessive levels of ROS and maintain cellular redox homeostasis (He et al. 2017). However, disruption of this balanced system and overwhelmed cellular ROS production lead to oxidative stress (Agarwal et al. 2006). Oxidative stress could arise from external and/or internal stressors such as heat stress (Alemu et al. 2018), inflammation (Mittal et al. 2014) and endoplasmic reticulum stress (Liu et al. 2018). Exposure of cells to oxidative stress has a negative impact on cell membrane permeability, mitochondrial activity and causes DNA damage (Kadenbach et al. 2004; Zhang et al. 2016) resulting in cell cycle arrest (Saeed-Zidane et al. 2017) and apoptosis (Sohel et al. 2019).

Mammalian cells respond to intra and extra signals by altering several transcription factors (TFs), that activate and/or inhibit genes involved in specific biological functions (Leite et al. 2017). Some of those TFs are believed to be involved in cellular defense mechanisms against oxidative stress (Mikaeili et al. 2016; Saeed-Zidane et al. 2017; Weng et al. 2016). In bovine embryos, the capability of *in vitro* produced embryos to survive under oxidative stress conditions relies on the activation of nuclear factor (erythroid-derived 2)-like 2 (NRF2 or NFE2L2) transcription factor at mRNA and protein

levels (Amin et al. 2014). NRF2 is responsible for the activation of cellular antioxidants machinery as has been previously reported in bovine granulosa cells (Saeed-Zidane et al. 2017) and preimplantation embryos (Amin et al. 2014). Moreover, NRF2 could be extracellularly released via exosomes and horizontally transferred to neighboring cells as a means of increasing their adaptive response against oxidative stress (Saeed-Zidane et al. 2017). On the other hand, NRF2 might particularly be involved in the regulation of other TFs network such as NOTCH (Zhao et al. 2016), sterol regulatory element-binding transcription factors (SREBFs) family (Amin et al. 2014; Kamisako et al. 2014) and Krüppel-like factor (KLFs) family (Jang et al. 2014; Zucker et al. 2014). Some of those TFs significantly contribute to the regulation of ovarian functions (Murta et al. 2015; Natesampillai et al. 2008). However, increasing intracellular ROS accumulation accompanied by overexpression of *NRF2* resulted in promoting cell death via regulation of KLF9 (Zucker et al. 2014). However, little is known regarding the regulatory role of the TFs in modulating bovine granulosa cells functions under oxidative stress conditions. Therefore, the current study aimed to investigate the expression patterns of stress-related TFs and their association with the *NRF2* signaling pathway in bovine granulosa cells exposed to oxidative stress.

2.3 Materials and methods

2.3.1 Experimental setup

To investigate the effect of H_2O_2 on the expression of the stress-related TFs and their regulation by the NRF2 signaling pathway in bovine granulosa cells, two independent experiments were conducted. In the first experiment, sub-confluent bovine granulosa cells were treated with 5 µM H_2O_2 for 40 min (Saeed-Zidane et al. 2017) to induce moderate oxidative stress. Thereafter, the culture media was replaced with fresh media and cells were incubated at 37 °C in 5% CO_2 for a further 24 h. The intracellular ROS accumulation, DNA fragmentation, mitochondrial activity, cell proliferation and lipid accumulation were assessed in the treated and control groups. Furthermore, cells of both groups were subjected to mRNA and protein levels quantification. Based on previous evidence and *in silico* analysis, the second experiment was conducted in order to figure out the cross-talk between NRF2 and candidate TFs. For that, sub-confluent bovine granulosa cells were transfected with 200 nM small interference RNA (siRNA) targeting NRF2. Twenty-four h later, cell proliferation and lipid accumulation analysis were

performed. In addition, the mRNA and protein levels of *NRF2* and the candidate TFs were investigated.

2.3.2 Culture of bovine granulosa cells

Bovine ovaries were obtained from a local abattoir (Düren, Germany) and transported in warm (37 °C) physiological saline (0.9% NaCl). Upon arrival, ovaries were washed three times with warm (37 °C) 0.9% NaCl, followed by washing with prewarmed (37 °C) 70% ethanol, and then washed with warm 0.9% NaCl. Granulosa cells were aspirated from small follicles (3-5 mm) in 15 mL tubes containing prewarmed (37 °C) phosphate-buffered saline, calcium- and magnesium-free (PBS-CMF) and then kept standing for 15 min at 37 °C to let the cellular debris settled down at the bottom. Subsequently, the supernatants containing the granulosa cells were collected and centrifuged at 750 g for 7 min to get granulosa cells pellets. Afterwards, the cell viability and concentration were checked under the microscope using trypan blue. Ultimately, a total of 2.5 x 10^5 cells per well was cultured in CytoOne® 24-well plate (Starlab GmbH, Hamburg, Germany) containing 600 µL DMEM/F-12 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin and incubated at 37 °C in 5% CO_2. Twenty-four h post-treatment, cells were subjected to various phenotypic and genotypic analysis.

2.3.3 Small interference RNA transfection

In order to investigate the cross-talk between NRF2 and the candidate TFs, the siRNA-mediated NRF2 knockdown was utilized. Briefly, sub-confluent granulosa cells were transfected with 200 nM siRNA targeting NRF2 (Exiqon, Vedbaek, Denmark) in the presence of Lipofectamine® 2000 (Invitrogen, Carlsbad, California, USA) as transfection reagent diluted in Opti-MEM I reduced-serum medium (Invitrogen, Carlsbad, California, USA). Afterwards, cells were incubated for 24 h in a humidified 5% CO_2 incubator at 37 °C. At the end of the incubation period, the culture media were removed and cells were used for further analysis.

2.3.4 Cell proliferation assay

A total of 1.5 x 10^4 cells was cultured in 96-well plate using the same procedure described above. After 24 h from the treatment of sub-confluent cells with either H_2O_2 or siRNA, the cell proliferation assay was performed using CCK-8 kits (Dojindo EU GmbH,

München, Germany) according to the manufacturer´s protocol. The proliferation rate was indicated by the absorbance at 450 nm wavelength using Synergy™ H1 Multi-Mode Reader (BioTek Germany, Bad Friedrichshall, Germany).

2.3.5 Intracellular ROS detection

To determine the effect of oxidative stress on intracellular ROS accumulation, granulosa cells were cultured in 96-well plate and then the sub-confluent cells were treated with H_2O_2 to induce oxidative stress. After 24 h, cells were co-incubated with FBS-free media containing 75 µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Invitrogen, Carlsbad, California, USA) for 20 min at 37 °C in 5% CO_2. Subsequently, the cells were washed twice with PBS-CMF and then the images were captured using a green-fluorescence filter of the inverted fluorescence microscope (Leica DM IRB, Leica, Wetzlar, Germany). The fluorescence intensity was quantified using ImageJ 1.48v (National Institutes of Health, Maryland, USA, http://imagej.nih.gov).

2.3.6 Mitochondrial activity assay

To assess the cellular mitochondrial activity in bovine granulosa cells exposed to oxidative stress conditions, a total of 4 x 104 live cells were seeded onto 8-well slide chamber according to the manufacturer´s protocol. Briefly, the granulosa cells were co-incubated with 200 nM MitoTracker red dye (MitoTracker® Red CMXRos, M7512; Invitrogen) for 30 min. Then, the cells were rinsed twice with PBS-CMF and subsequently fixed with 4% paraformaldehyde overnight at 4 °C. Fixed cells were mounted with vectashield mounting medium containing DAPI. Finally, the images were acquired using confocal microscopy CLSM LSM-780 and the fluorescent intensity was analyzed with ImageJ 1.48v software (National Institutes of Health, Maryland, USA, http://imagej.nih.gov).

2.3.7 Lipid accumulation assay

To investigate the cellular accumulation of lipid, the cells were cultured into a 96-well plate and treated with either H_2O_2 or siRNA. Twenty-four h post-treatment, plates containing granulosa cells were gently rinsed with 50 µL of PBS-CMF. Subsequently, cells were fixed using 4% paraformaldehyde overnight at 4 °C and then subjected to lipid accumulation assay. Briefly, a working solution was prepared by mixing three parts (30

mL) of oil red O (Sigma-Aldrich Chemie GmbH, Munich, Germany) from stock solution with 2 parts (20 mL) deionized water and then stored at room temperature for 10 min followed by filtration via filter paper. After removing the 4% paraformaldehyde, each well was gently rinsed by sterile water followed by co-incubation with 50 µL of 60% isopropanol at room temperature for 5 min. Afterwards, the isopropanol was poured off and 100 µL working solution of oil red O was pipetted to each well and left for 5 min. Following this, cells were rinsed with tap water and the images were captured using an inverted microscope with 40x magnification.

2.3.8 DNA fragmentation

The fragmented DNA was isolated according to the Abcam protocol (Cambridge, UK). Briefly, the floated and adhered cells were collected and then lysed in 500 µL of 10 mM Tris (pH 7.4), 5 mM EDTA, 0.2% Triton-X100 followed by vortexing for several times. The lysate was incubated on ice for 30 min followed by centrifugation at 27,000 g for 30 min. Subsequently, the supernatants were divided into two parts and 50 µL ice-cold 5 M NaCl was added to each part coupled with several times of vortexing. Subsequently, a mixture containing 600 µL ethanol and 150 µL 3 M sodium acetate (pH 5.2) was added to each aliquot with gentle mixing by pipetting and then incubated at -80 °C for 1 h followed by centrifugation for 20 min at 20,000 g. After discarding the supernatant, the pellets were pooled together and re-dissolved in 10 mM Tris and 5 mM EDTA accompanied by adding DNase-free RNase 10 mg/mL and then incubated in a water bath at 37 °C for 5 h. Afterwards, a combination of 25 µL proteinase K at 20 mg/mL (Qiagen GmbH, Hilden, Germany) and 40 µL buffer (100 mM Tris pH 8.0, 100 mM EDTA and 250 mM NaCl) was added and subsequently incubated overnight at 65 °C. Thereafter, the DNA was extracted using phenol:chloroform:isoamyl alcohol mixture (25:24:1) (Biomol GmbH, Hamburg, Germany) and then precipitated using ethanol. The supernatant was carefully removed followed by air drying. The pellets were resuspended in 15 µL 1x TE buffer (Carl Roth GmbH, Karlsruhe, Germany). Finally, the samples were electrophoretically separated using 2% agarose gel containing ethidium bromide and then visualized using ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany).

2.3.9 RNA isolation and cDNA synthesis

Total RNA from granulosa cells was extracted using the miRNeasy® mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Before starting the total RNA extraction, granulosa cells were washed using 1x PBS to remove remaining culture medium. At the end of the extraction protocol, trapped RNAs in the membrane of spin-column were eluted using 30 µL RNase-free water. The RNA concentration was assessed using NanoDrop 8000 UV-Vis Spectrophotometers (Thermo Scientific, Wilmington, USA). Equal amounts of total RNA were reverse transcribed using RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Schwerte, Germany). Briefly, a combination from oligo-dT and random primer was added to each sample followed by incubation at 65 °C for 5 min. After incubation time, a mixture containing 1 µL of RiboLock RNase inhibitor, 2 µL of 10 mM dNTP mix, 4 µL of 5x reaction buffer and 2 µL of MMuLV reverse transcripts was added to each sample. Afterwards, samples were incubated at 25 °C for 5 min, then 37 °C for 60 min followed by 70 °C for 5 min to terminate the reaction. Ultimately, the cDNA was kept at -20 °C for further analysis.

2.3.10 Real-Time Quantitative PCR

The expression levels of candidate TFs (NRF2, NOTCH1, SREBF1, SREBF2, KLF4, KLF9, FOS, FOSB, IRF7, E2F1 and E2F4), the KLF4 downstream target genes (CCNB1, CCND2 and BAX), endoplasmic reticulum stress marker (Grp78, Grp94 and Calpain1), cell differentiation markers (CYP11A1, CYP19A1, STAR, INHBa, EGFR and FOXO1) and apoptosis-related genes (BCL2L1 and CASP3) were quantified using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany) in Applied Biosystem® StepOnePlus™ (Applied biosystems, Foster City, CA, USA). The primers were designed based on the primer3 online tool (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 1). Subsequently, the specificity of the primers was confirmed by sequencing analysis using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter GmbH, Krefeld, Germany). The mRNAs expression data were analyzed using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schmittgen 2001) and the geometric mean of the expression level of ACTB and GAPDH was used for normalization after checking the expression stability with NormFinder software (Andersen et al. 2004).

2.3.11 Immunoblotting

Total protein of granulosa cells was isolated using 1x passive lysis buffer (Promega GmbH, Mannheim, Germany) and the protein concentration was determined using Coomassie Protein Assay Reagent (Life Technologies GmbH, Darmstadt, Germany). Thirty-five µg of protein was loaded onto 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred from the gel into nitrocellulose membrane (Bio-Rad Laboratories GmbH, Germany) using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories GmbH, Germany). Afterwards, the membrane was blocked in Roti®-Block (Carl Roth GmbH, Germany) for 1 h at room temperature. The membrane was incubated overnight at 4 °C with polyclonal primary antibodies specific for KLF4 (1:100), Grp78 (1:300), AMPKa1/2 (1:350), StAR (1:250) and ACTB (1:500) mouse monoclonal antibody monoclonal (Santa Cruz Biotechnology Inc, Germany). After the expiration of the incubation period, the membrane was incubated with goat anti-rabbit or goat anti-mouse (1:5000; Santa Cruz Biotechnology Inc, Germany) according to the candidate primary antibody for 1 h at room temperature. The proteins were visualized using Clarity™ Western ECL Substrate (Bio-Rad Laboratories Inc, USA). The images were captured using the ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany). Subsequently, the membrane was stripped using middle stripping buffer in order to remove primary and secondary antibodies followed by blocking and incubation with another antibody using the same protocol.

2.3.12 Immunocytochemistry

Quantification and localization of NRF2 and KLF4 proteins were conducted using the immunocytochemistry technique. For this, granulosa cells were seeded into 8-well slide chamber and subsequently treated with either H_2O_2 or siRNA. Twenty-four h post-treatment, granulosa cells were fixed using 4% paraformaldehyde and kept at 4 °C overnight. Subsequently, the cells were rinsed three times with PBS-CMF and then incubated with 0.3% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min followed by blocking with 3% donkey serum for 1 h at room temperature. Afterwards, the cells were co-incubated with primary antibody for NRF2 (1:100) or KLF4 (1:100) at 4 °C overnight. Thereafter, the cells were washed three times with PBS-CMF followed by co-incubation with fluorescent secondary antibody (Lifespan Biosciences, Alexa fluor goat anti-rabbit 1:350) for 3 h at 37 °C in dark. Thereafter, the cells were

washed three times with PBS-CMF and subsequently mounted with vectashield mounting medium containing DAPI. Eventually, the cells were visualized under confocal microscopy CLSM LSM-780 and the fluorescence intensity of proteins was analyzed using ImageJ 1.48v software (National Institutes of Health, Maryland, USA, http://imagej.nih.gov).

2.3.13 Statistical analysis

Data were represented as means $\pm$ SEM from at least three independent biological replicates. Data were statistically analyzed with student's two-tailed t-test using GraphPad Prism software version 7 (GraphPad Software, La Jolla, California, USA). The difference between means was considered significant when *P* value less than *0.05*.

2.4 Results

2.4.1   Oxidative stress impaired granulosa cell functions

Bovine granulosa cells treated with H_2O_2 exhibited shrunken cell shape and decreased confluency compared to untreated counterparts (Figure 2.1). Moreover, exposure of cells to H_2O_2 increased ($P \leq 0.01$) the intracellular ROS levels and decreased ($P \leq 0.001$) mitochondrial activity compared to the control counterparts (Figure 2.2a and b). Moreover, the higher intracellular ROS accumulation in H_2O_2-challenged cells was accompanied by the induction of DNA fragmentation (Figure 2.3). Overall, these results indicated that bovine granulosa cells treated with H_2O_2 resulted in induction of oxidative stress.

2.4.2 H_2O_2-induced oxidative stress coupled with endoplasmic reticulum stress

The mRNA expression level of glucose-regulated protein (Grp) 78 and Grp94 was significantly ($P \leq 0.01$ and $P \leq 0.05$, respectively) higher in H_2O_2-treated cells compared to control counterparts (Figure 2.4a). Furthermore, the protein level of Grp78 was in agreement with its mRNA expression level (Figure 2.4b). However, the expression level of Calpain1 did not show any significant differences.

2.4.3 H₂O₂-induced oxidative stress altered the expression pattern of candidate TFs

The temporal expression level of the candidate TFs which have pivotal roles in stress response (NRF2, c-FOS and FOSB), apoptosis, differentiation and proliferation (KLF4, KLF6, KLF9 and NOTCH1), cell cycle (E2F1 and E2F4), fatty acids and cholesterol biosynthesis (SREBF1 and SREBF2) and immune response (IRF7) was quantified. Upon oxidative stress induction, the expression pattern of NRF2 was significantly increased ($P \leq 0.01$) in the H_2O_2-challenged group compared to the unchallenged control group (Figure 2.5a). Similarly, the mRNA transcript level of NOTCH1, c-FOS, SREBF1, SREBF2, KLF6, KLF9 and E2F1 was significantly higher ($P \leq 0.05$) in H_2O_2-treated group (Figure 2.5c). Contrary to this, the expression of KLF4 and IRF7 was significantly lower ($P \leq 0.05$) in H_2O_2-treated granulosa cells compared to the untreated control group (Figure 2.5d). However, no significant differences were observed in the expression of E2F4 and FOSB (Figure 2.5d).

2.4.4 Oxidative stress influenced the protein level of NRF2 and KLF4

To confirm the temporal expression of mRNA, the protein level of one of upregulated candidate TFs (NRF2) and other downregulated (KLF4) was assessed using immunocytochemistry assay. Similar to mRNA expression, the protein abundance of NRF2 was significantly upregulated ($P \leq 0.001$) in H_2O_2-challenged cells compared to control counterparts (Figure 2.5b). Furthermore, the NRF2 protein was found to be localized in the nucleus of treated cells. However, the protein abundance of KLF4 was significantly lower ($P \leq 0.001$) in the H_2O_2-challenged cells (Figure 2.6a).

2.4.5 Aberrations of KLF4 downstream genes under oxidative stress condition

The downregulation of KLF4 in the H_2O_2-treated group was accompanied by a significant reduction ($P \leq 0.01$) of CCNB1 (Figure 2.6b). However, the mRNA expression of CCND2 and BAX was not statistically significantly differed between H_2O_2-treated and untreated groups (Figure 2.6b).

2.4.6 Oxidative stress induced lipid accumulation

In addition to KLF4, to confirm whether the increased expression of TFs involved in fatty acids and cholesterol biosynthesis (SREBF1 and SREBF2) under oxidative stress

condition altered granulosa cell functions, the expression of upstream genes of SREBFs and cellular lipid accumulation were investigated. The results showed a significant increment ($P \leq 0.05$) in mRNA expression of AMPKa1 (Figure 2.7a). Additionally, the AMPKa1/2 protein level was upregulated in H_2O_2-challenged cells (Figure 2.7b). Consequently, the H_2O_2-challenged cells exhibited higher lipid accumulation compared to control ones (Figure 2.7c).

2.4.7   Oxidative stress induced differentiation marker genes

To clarify whether the exposure of bovine granulosa cells to oxidative stress affected their ability to differentiate, the expression pattern of differentiation marker genes was investigated. The mRNA expression of CYP11A1, CYP19A1 and STAR was significantly upregulated ($P \leq 0.001$, $P \leq 0.01$, and $P \leq 0.01$, respectively) upon exposure of granulosa cells to H_2O_2 (Figure 2.8a). In addition, the protein abundance of StAR showed upregulation in challenged cells (Figure 2.8b). Moreover, the expression pattern of EGFR was significantly upregulated ($P \leq 0.01$) in H_2O_2-treated group compared to untreated counterparts. However, no significant differences were noticed in the mRNA expression level of Inhibin, Beta A (INHBa) and Forkhead box O1 (FOXO1) between treated and untreated groups (Figure 2.8a).

2.4.8   Oxidative stress induced the expression of the pro-apoptotic gene and subsequently impaired granulosa cells proliferation

Unlike BAX, the mRNA level of CASP3, a pro-apoptotic gene, was significantly increased ($P \leq 0.05$) in H_2O_2-challenged cells (Figure 2.9a). However, the expression of the anti-apoptotic marker gene (BCL2L1) was significantly decreased ($P \leq 0.05$) under H_2O_2 challenge. On the other hand, the mRNA expression level of the proliferation marker gene (PCNA) was significantly decreased ($P \leq 0.01$) under oxidative stress condition (Figure 2.9b and d). Accordingly, a significant reduction in cell proliferation rate was observed upon exposure of granulosa cells to H_2O_2 challenge (Figure 2.9c).

2.4.9   Selective knockdown of NRF2 altered the mRNA expression pattern of stress-related candidate TFs

The granulosa cells transfected with siRNA targeting NRF2 exhibited significantly lower expression of NRF2 ($P \leq 0.001$, Figure 2.10). Upon NRF2 Knockdown, the mRNA

expression of KLF9, c-FOS, NOTCH1 and SREBF2 was significantly downregulated ($P \leq 0.05$; Figure 2.10c). Interestingly, the relative mRNA expression of KLF4 was significantly increased ($P \leq 0.001$) with 5-fold. However, the mRNA expression levels of E2F1 and SREBF1 were not significantly altered.

2.4.10 NRF2 knockdown altered the KLF4 downstream genes and reduced cell proliferation

Targeted suppression of NRF2 increased the protein level of KLF4 (Figure 2. 11a). Next, to demonstrate the effect of NRF2 knockdown induced expression of KLF4 on granulosa cell functions, the expression pattern of its downstream genes and cell proliferation were investigated. Consequently, the mRNA expression of CCND2 was significantly upregulated ($P \leq 0.05$; Figure 2.11b). Intriguingly, the expression pattern of BAX was significantly upregulated ($P \leq 0.01$) accompanied by a reduction in cell proliferation rate (Figure 2. 11c).

2.4.11 NRF2 knockdown altered the expression pattern of genes associated with differentiation and lipid accumulation

Since the upregulation of TFs involved in fatty acids and cholesterol biosynthesis as well as differentiation was noticed in granulosa cells expressed NRF2, the impact of NRF2 knockdown on the expression of differentiation genes and the amount of lipid accumulation was evaluated. Accordingly, the mRNA expression level of the CYP11A1 transcript was reduced ($P \leq 0.05$) in siRNA-targeting NRF2 transfected cells (Figure 2.12a) and the mRNA level of INHBa was significantly increased ($P \leq 0.01$). On the other hand, the NRF2 knockdown resulted in dysregulation of fatty acids and cholesterol biosynthesis transcription factors, which was confirmed by lower lipid accumulation under inhibition of NRF2 (Figure 2.12b).

2.5 Discussion

Follicle containing oocyte surrounded by multiple layers of granulosa and theca cells is the functional unit of the mammalian ovary. In fact, granulosa cells provide oocyte with essential nutrients (Dzafic et al. 2013) and contribute to the production of steroid hormones (Wen et al. 2010). During the bovine follicular wave, one of the growing follicles becomes dominant and is subsequently ovulated, while the other follicles

undergo atresia (Ginther et al. 2000; Hirshfield 1991; Shen et al. 2012), which begins from granulosa cells (Inoue et al. 2011; Jiang et al. 2003).

The elevated level of ROS during follicular atresia (Hennet et al. 2013) and the ovulation process (Shkolnik et al. 2011) is a common phenomenon in the ovarian environment. Moreover, oxidative stress is occurred as a result of exposure to stress predisposing factors including diseases (Lai et al. 2018), heat stress (Alemu et al. 2018; Li et al. 2016a) and food toxicity (Qin et al. 2015), which lead to compromise granulosa cells functions and other interacting cells in ovarian follicle (Li et al. 2016b; Zhang et al. 2016).

Various concentrations of H_2O_2 have been used to induce *in vitro* oxidative stress (Saeed-Zidane et al. 2017; Sohel et al. 2019; Weng et al. 2016; Zhang et al. 2016). Based on our previous work (Saeed-Zidane et al. 2017), 5 µM H_2O_2 was selected to induce moderate oxidative stress in bovine granulosa cells in the current study.

In the current study, exposure of cells to H_2O_2 induced oxidative stress through increasing intracellular ROS accumulation (Figure 2.2a), which is similar to previous reports (Saeed-Zidane et al. 2017; Sohel et al. 2019; Zhang et al. 2016). Upon increasing intracellular ROS accumulation, we found that the mitochondrial activity was reduced in H_2O_2-challenged cells (Figure 2.2b) indicating the impairment of the mitochondrial integrity, which in turn altered other biological functions like metabolism (Kadenbach et al. 2004), apoptosis (Yang et al. 1997) and proliferation as well as cell cycle (Antico Arciuch et al. 2012; Saeed-Zidane et al. 2017). Besides the compromised mitochondrial activity, the ROS can bind to bioactive molecules such as DNA and subsequently cause DNA damage (Kadenbach et al. 2004; Zhang et al. 2016). Similarly, the elevated level of ROS in the H_2O_2-challenged cells was accompanied by higher DNA fragmentation (Figure 2.3). Furthermore, we found that the H_2O_2-induced oxidative stress contributed to induction of endoplasmic reticulum stress, which was confirmed by the upregulation of endoplasmic reticulum marker genes (Grp78 and Grp94) mRNA and consequentially higher protein level of Grp78 (Figure 2.4). Grp78 and Grp94 contribute to the maintenance of endoplasmic reticulum homeostasis and induction of apoptosis via activation of caspase under endoplasmic reticulum stress (Liu et al. 2011). Matched with current results, we have previously shown that granulosa cells exposed to heat stress showed upregulation of endoplasmic reticulum stress marker genes (Alemu et al. 2018).

In ovarian cells, KLFs' family contributes significantly to various functions including cell proliferation, differentiation and apoptosis (Jo et al. 2004; Kezele et al. 2005; Simmen et

al. 2015). In the same line to this, the expression of KLF4 and KLF13 mRNA is regulated by luteinizing hormone (LH) or/and insulin-like growth factor 1 (IGF1) in follicular granulosa cells (Natesampillai et al. 2008), while the dysregulation of KLF2 and KLF4 expression was noticed in polycystic ovary syndrome (PCOS) compared with normal ovaries (Jansen et al. 2004). The aberrant expression of KLF2, KLF4, KLF5, KLF6 and KLF9 was found in ovarian cancer cells (DiFeo et al. 2006; Dong et al. 2013; Zhang et al. 2015). Accordingly, our results revealed that the mRNA level of KLF6 and KLF9 was upregulated under oxidative stress conditions (Figure 2.5c). The upregulation of KLF9 could be attributed to the higher expression of NRF2 and subsequently induce apoptosis (Zucker et al. 2014).

Multiple signaling pathways including TFs contribute to induction of apoptosis. Supporting this notion, the E2F1, which is one of E2Fs TFs family involved in cell cycle regulation and apoptosis (Crosby and Almasan 2004), was increased in H_2O_2-treated granulosa cells (Figure 2.5c). Consistently, it had been shown that the upregulation of E2F1 was accompanied by increasing of apoptosis in different cell types (Ginsberg 2002), which was evidenced by increasing DNA fragmentation in the present study (Figure 2.3). Additionally, we found that the mRNA transcript of Fos proto-oncogene, AP-1 transcription factor subunit (c-FOS) was significantly increased upon oxidative stress induction (Figure 2.5c). The c-FOS is a member of the activator protein-1 (AP-1) family including c-Jun, JunB, JunD, c-Fos, FRA1 and FRA2, which is regulated by c-Jun NH2-terminal kinase (JNK) AP-1 (Yin et al. 2009). Under oxidative stress conditions, the upregulation of JNK triggered apoptosis via FOXO1 (Weng et al. 2016), which in turn induced c-FOS expression (Zhang et al. 2007). These findings were not in agreement with our current results under oxidative stress conditions (Figure 2.5c).

Unexpectedly, the mRNA expression of KLF4 was significantly decreased under oxidative stress conditions (Figure 2.5c), which subsequently confirmed by its protein level (Figure 2.6a). The reduction of KLF4 mRNA and protein levels attenuated the mRNA expression of its target genes (Figure 2.6b), which were found to be involved in the regulation of apoptosis (Li et al. 2015) and cell cycle (Choi and Roh 2019). Our previous study showed downregulation of CCND2 and subsequently cell cycle arrest at the G_2/M phase in H_2O_2-treated granulosa cells (Saeed-Zidane et al. 2017). These results were conflicted with overexpression of KLF4 in mice granulosa cells exposed to 100 µM

H_2O_2 (Xu et al. 2017), which indicates that the expression of KLF4-induced apoptosis is expressed in H_2O_2 dose-dependent manner.

From another point of view, the reduction of KLF4 expression was found to be indirectly regulated by the higher expression of SREBF2 under oxidative stress condition in endothelial cells (Chen et al. 2015). SREBF1 and SREBF2 are TFs involved in regulation of fatty acids and cholesterol biosynthesis (Shimano 2001). Similarly, our results showed that SREBF1 and SREBF2 were found to be upregulated in granulosa cells exposed to oxidative stress conditions (Figure 2.5c), which was coupled with a reduction of KLF4 expression. The expression of SREBF1 was found to be more abundant in bovine embryos exposed to oxidative stress-induced NRF2 expression (Amin et al. 2014). Furthermore, the elevated ROS-induced endoplasmic reticulum stress is responsible for increasing the SREBF2-cleavage (Colgan et al. 2007), which is in the same line of the current study. To validate that, the lipid accumulation assay was performed and the results showed higher lipid content of granulosa cells in response to H_2O_2-induced oxidative stress (Figure 2.7c). The higher lipid accumulation may be attributed to apoptosis and differentiation of granulosa cells (Regan et al. 2018), as evidenced from higher expression of differentiation marker genes (Figure 2.8) and apoptosis (Figure 2.9). Additionally, the oxidative stress-induced apoptosis was confirmed by a reduction in the expression of cell proliferation marker genes (Figure 2.9b) and cell proliferation rate (Figure 2.9c). Our results were in agreement with Saeed-Zidane et al. (2017), who showed that exposure of granulosa cells to oxidative stress led to the upregulation of apoptosis and differentiation marker genes. This could indicate the significant contribution of ROS in granulosa cells transdifferentiation (Shkolnik et al. 2011).

There is clear evidence that the NOTCH signaling pathway is implicated in ovarian follicular development, through the spatial and temporal regulation of its associated genes, during different stages of follicular growth and selection (Murta et al. 2015; Vanorny et al. 2014; Zhang et al. 2011). Moreover, it is also involved in corpus leutum steroidogenesis and regression (Murta et al. 2015). Additionally, the NOTCH signaling acts as a suppressor of apoptosis signal-regulating kinase 1 (ASK1) 1 and consequently p38 MAPK signaling pathway (Mo et al. 2013). Accordingly, our data showed an upregulation of NOTCH1 transcript in H_2O_2-treated group (Figure 2.5c). In this regard, the NOTCH signaling was found to be upregulated in granulosa cells of preantral and antral follicles (Jing et al. 2017), which indicates the vital role of this pathway in the

regulation of granulosa cell functions during follicle development (Jing et al. 2017). However, the NOTCH signaling pathway was also found to be involved in suppression of AP-1 (Murthy et al. 2012), which is inconsistence with our findings (Figure 2.5c). Moreover, it was reported that the expression of *NOTCH1* was underwent induction of NRF2 to protect cells from apoptosis (Zhao et al. 2016), which revealed the role of NRF2 in the regulation of other TFs.

To investigate the cross-talk between the NRF2 signaling pathway and candidate TFs, the NRF2 gene was selectively knocked down using siRNA technology. Our results revealed that the inhibition of NRF2 led to reducing the expression of several candidate TFs including KLF9, c-FOS, NOTCH1 and SREBF2 (Figure 2.10c). However, the expression of KLF4 was significantly increased (Figure 2.10c), which was guaranteed by the induction of its protein level (Figure 2.11a). Subsequently, the downstream target genes of KLF4 were increased (Figure 2.11b), which in turn resulted in a reduction of granulosa cells proliferation (Figure 2.11c). These results may indicate the role of NRF2 in suppressing apoptosis via inhibition of KLF4-mediated BAX (Li et al. 2015). Additionally, the dysregulation of SREBFs' levels resulted in reducing lipid accumulation upon NRF2 knockdown. This led to a reduction of CYP11A1 differentiation marker gene (Figure 2.12a), which demonstrated the vital role of NRF2 in harmonic with other TFs in regulation of granulosa cells differentiation. The present study opened a new window in understanding the potential role of NRF2 and other TFs in regulation of granulosa cell functions under oxidative stress conditions.

To sum up, we found that granulosa cells exposed to oxidative stress showed higher intracellular ROS accumulation accompanied by increased DNA fragmentation and reduced mitochondrial activity. Therefore, the cells activated the NRF2-mediated oxidative stress response in parallel with other TFs involved in apoptosis and differentiation, which was remarkably observed in the reduction of cell proliferation and induction of lipid accumulation. This effect was reciprocally observed with NRF2 knockdown along with a reduction of cell proliferation. Taken together, the NRF2 signaling pathway in cooperation with other stress-related TFs plays a vital role in the oxidative stress response through the regulation of cell apoptosis and differentiation in bovine granulosa cells, which could occur during follicular development.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

2.6 References

Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG (2006) Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 86:503-512

Alemu TW, Pandey HO, Salilew Wondim D, Gebremedhn S, Neuhof C, Tholen E, Holker M, Schellander K, Tesfaye D (2018) Oxidative and endoplasmic reticulum stress defense mechanisms of bovine granulosa cells exposed to heat stress. Theriogenology 110:130-141

Amin A, Gad A, Salilew-Wondim D, Prastowo S, Held E, Hoelker M, Rings F, Tholen E, Neuhoff C, Looft C, Schellander K, Tesfaye D (2014) Bovine embryo survival under oxidative-stress conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway. Mol Reprod Dev 81:497-513

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245-5250

Antico Arciuch VG, Elguero ME, Poderoso JJ, Carreras MC (2012) Mitochondrial regulation of cell cycle and proliferation. Antioxid Redox Signal 16:1150-1180

Chen Z, Wen L, Martin M, Hsu CY, Fang L, Lin FM, Lin TY, Geary MJ, Geary GG, Zhao Y, Johnson DA, Chen JW, Lin SJ, Chien S, Huang HD, Miller YI, Huang PH, Shyy JY (2015) Oxidative stress activates endothelial innate immunity via sterol regulatory element binding protein 2 (SREBP2) transactivation of microRNA-92a. Circulation 131:805-814

Choi H, Roh J (2019) Role of Klf4 in the regulation of apoptosis and cell cycle in rat granulosa cells during the periovulatory period. Int J Mol Sci 20:

Colgan SM, Tang D, Werstuck GH, Austin RC (2007) Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2. Int J Biochem Cell Biol 39:1843-1851

Crosby ME, Almasan A (2004) Opposing roles of E2Fs in cell proliferation and death. Cancer Biol Ther 3:1208-1211

Di Meo S, Reed TT, Venditti P, Victor VM (2016) Role of ROS and RNS sources in physiological and pathological conditions. Oxid Med Cell Longev 2016:1245049

DiFeo A, Narla G, Hirshfeld J, Camacho-Vanegas O, Narla J, Rose SL, Kalir T, Yao S, Levine A, Birrer MJ, Bonome T, Friedman SL, Buller RE, Martignetti JA (2006)

Roles of KLF6 and KLF6-SV1 in ovarian cancer progression and intraperitoneal dissemination. Clin Cancer Res 12:3730-3739

Dong Z, Yang L, Lai D (2013) KLF5 strengthens drug resistance of ovarian cancer stem-like cells by regulating survivin expression. Cell Prolif 46:425-435

Dzafic E, Stimpfel M, Virant-Klun I (2013) Plasticity of granulosa cells: on the crossroad of stemness and transdifferentiation potential. J Assist Reprod Genet 30:1255-1261

Ginsberg D (2002) E2F1 pathways to apoptosis. FEBS Lett 529:122-125

Ginther OJ, Bergfelt DR, Kulick LJ, Kot K (2000) Selection of the dominant follicle in cattle: role of estradiol. Biol Reprod 63:383-389

He L, He T, Farrar S, Ji L, Liu T, Ma X (2017) Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. Cell Physiol Biochem 44:532-553

Hennet ML, Yu HY, Combelles CMH (2013) Follicular fluid hydrogen peroxide and lipid hydroperoxide in bovine antral follicles of various size, atresia, and dominance status. J Assist Reprod Gen 30:333-340

Hirshfield AN (1991) Development of follicles in the mammalian ovary. Int Rev Cytol 124:43-101

Inoue N, Matsuda F, Goto Y, Manabe N (2011) Role of cell-death ligand-receptor system of granulosa cells in selective follicular atresia in porcine ovary. J Reprod Dev 57:169-175

Jancar N, Kopitar AN, Ihan A, Virant Klun I, Bokal EV (2007) Effect of apoptosis and reactive oxygen species production in human granulosa cells on oocyte fertilization and blastocyst development. J Assist Reprod Genet 24:91-97

Jang J, Wang Y, Kim HS, Lalli MA, Kosik KS (2014) Nrf2, a regulator of the proteasome, controls self-renewal and pluripotency in human embryonic stem cells. Stem Cells 32:2616-2625

Jansen E, Laven JS, Dommerholt HB, Polman J, van Rijt C, van den Hurk C, Westland J, Mosselman S, Fauser BC (2004) Abnormal gene expression profiles in human ovaries from polycystic ovary syndrome patients. Mol Endocrinol 18:3050-3063

Jiang JY, Cheung CK, Wang Y, Tsang BK (2003) Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. Front Biosci 8:d222-237

Jing J, Jiang X, Chen J, Yao X, Zhao M, Li P, Pan Y, Ren Y, Liu W, Lyu L (2017) Notch signaling pathway promotes the development of ovine ovarian follicular granulosa cells. Anim Reprod Sci 181:69-78

Jo M, Gieske MC, Payne CE, Wheeler-Price SE, Gieske JB, Ignatius IV, Curry TE, Jr., Ko C (2004) Development and application of a rat ovarian gene expression database. Endocrinology 145:5384-5396

Kadenbach B, Arnold S, Lee I, Huttemann M (2004) The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. Biochim Biophys Acta 1655:400-408

Kamisako T, Tanaka Y, Kishino Y, Ikeda T, Yamamoto K, Masuda S, Ogawa H (2014) Role of Nrf2 in the alteration of cholesterol and bile acid metabolism-related gene expression by dietary cholesterol in high fat-fed mice. J Clin Biochem Nutr 54:90-94

Kezele PR, Ague JM, Nilsson E, Skinner MK (2005) Alterations in the ovarian transcriptome during primordial follicle assembly and development. Biol Reprod 72:241-255

Lai Q, Xiang W, Li Q, Zhang H, Li Y, Zhu G, Xiong C, Jin L (2018) Oxidative stress in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with polycystic ovary syndrome. Front Med 12:518-524

Leite RF, Annes K, Ispada J, de Lima CB, Dos Santos EC, Fontes PK, Nogueira MFG, Milazzotto MP (2017) Oxidative stress alters the profile of transcription factors related to early development on in vitro produced embryos. Oxid Med Cell Longev 2017:1502489

Li J, Gao H, Tian Z, Wu Y, Wang Y, Fang Y, Lin L, Han Y, Wu S, Haq I, Zeng S (2016a) Effects of chronic heat stress on granulosa cell apoptosis and follicular atresia in mouse ovary. J Anim Sci Biotechnol 7:57

Li L, Wu J, Luo M, Sun Y, Wang G (2016b) The effect of heat stress on gene expression, synthesis of steroids, and apoptosis in bovine granulosa cells. Cell Stress Chaperones 21:467-475

Li W, Jiang Z, Li T, Wei X, Zheng Y, Wu D, Yang L, Chen S, Xu B, Zhong M, Jiang J, Hu Y, Su H, Zhang M, Huang X, Geng S, Weng J, Du X, Liu P, Li Y, Liu H, Yao Y, Li P (2015) Genome-wide analyses identify KLF4 as an important negative

regulator in T-cell acute lymphoblastic leukemia through directly inhibiting T-cell associated genes. Mol Cancer 14:26

Liu H, Zhang X, Zhang S, Huang H, Wu J, Wang Y, Yuan L, Liu C, Zeng X, Cheng X, Zhuang D, Zhang H (2018) Oxidative stress mediates microcystin-LR-induced endoplasmic reticulum stress and autophagy in KK-1 cells and C57BL/6 mice ovaries. Front Physiol 9:1058

Liu JF, Fong YC, Chang KW, Kuo SC, Chang CS, Tang CH (2011) FPTB, a novel CA-4 derivative, induces cell apoptosis of human chondrosarcoma cells through mitochondrial dysfunction and endoplasmic reticulum stress pathways. J Cell Biochem 112:453-462

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods 25:402-408

Mikaeili S, Rashidi BH, Safa M, Najafi A, Sobhani A, Asadi E, Abbasi M (2016) Altered FoxO3 expression and apoptosis in granulosa cells of women with polycystic ovary syndrome. Arch Gynecol Obstet 294:185-192

Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB (2014) Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal 20:1126-1167

Mo JS, Yoon JH, Ann EJ, Ahn JS, Baek HJ, Lee HJ, Kim SH, Kim YD, Kim MY, Park HS (2013) Notch1 modulates oxidative stress induced cell death through suppression of apoptosis signal-regulating kinase 1. Proc Natl Acad Sci U S A 110:6865-6870

Murta D, Batista M, Silva E, Trindade A, Mateus L, Duarte A, Lopes-da-Costa L (2015) Differential expression of Notch component and effector genes during ovarian follicle and corpus luteum development during the oestrous cycle. Reprod Fertil Dev 27:1038-1048

Murthy A, Shao YW, Narala SR, Molyneux SD, Zuniga-Pflucker JC, Khokha R (2012) Notch activation by the metalloproteinase ADAM17 regulates myeloproliferation and atopic barrier immunity by suppressing epithelial cytokine synthesis. Immunity 36:105-119

Natesampillai S, Kerkvliet J, Leung PC, Veldhuis JD (2008) Regulation of Kruppel-like factor 4, 9, and 13 genes and the steroidogenic genes LDLR, StAR, and CYP11A in ovarian granulosa cells. Am J Physiol Endocrinol Metab 294:E385-391

Qin X, Cao M, Lai F, Yang F, Ge W, Zhang X, Cheng S, Sun X, Qin G, Shen W, Li L (2015) Oxidative stress induced by zearalenone in porcine granulosa cells and its rescue by curcumin in vitro. PLoS One 10:e0127551

Rajani S, Chattopadhyay R, Goswami SK, Ghosh S, Sharma S, Chakravarty B (2012) Assessment of oocyte quality in polycystic ovarian syndrome and endometriosis by spindle imaging and reactive oxygen species levels in follicular fluid and its relationship with IVF-ET outcome. J Hum Reprod Sci 5:187-193

Regan SLP, Knight PG, Yovich JL, Leung Y, Arfuso F, Dharmarajan A (2018) Granulosa cell apoptosis in the ovarian follicle-a changing view. Front Endocrinol (Lausanne) 9:61

Roche JF, Boland MP (1991) Turnover of dominant follicles in cattle of different reproductive states. Theriogenology 35:81–90

Saeed-Zidane M, Linden L, Salilew-Wondim D, Held E, Neuhoff C, Tholen E, Hoelker M, Schellander K, Tesfaye D (2017) Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress. PLoS One 12:e0187569

Shen M, Lin F, Zhang J, Tang Y, Chen WK, Liu H (2012) Involvement of the up-regulated FoxO1 expression in follicular granulosa cell apoptosis induced by oxidative stress. J Biol Chem 287:25727-25740

Shimano H (2001) Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. Prog Lipid Res 40:439-452

Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N (2011) Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci U S A 108:1462-1467

Simmen RC, Heard ME, Simmen AM, Montales MT, Marji M, Scanlon S, Pabona JM (2015) The Kruppel-like factors in female reproductive system pathologies. J Mol Endocrinol 54:R89-R101

Sohel MMH, Akyuz B, Konca Y, Arslan K, Sariozkan S, Cinar MU (2019) Oxidative stress modulates the expression of apoptosis-associated microRNAs in bovine granulosa cells in vitro. Cell Tissue Res

Tanabe M, Tamura H, Taketani T, Okada M, Lee L, Tamura I, Maekawa R, Asada H, Yamagata Y, Sugino N (2015) Melatonin protects the integrity of granulosa cells by reducing oxidative stress in nuclei, mitochondria, and plasma membranes in mice. J Reprod Dev 61:35-41

Wen X, Li D, Tozer AJ, Docherty SM, Iles RK (2010) Estradiol, progesterone, testosterone profiles in human follicular fluid and cultured granulosa cells from luteinized pre-ovulatory follicles. Reprod Biol Endocrinol 8:117

Weng Q, Liu Z, Li B, Liu K, Wu W, Liu H (2016) Oxidative stress induces mouse follicular granulosa cells apoptosis via JNK/FoxO1 pathway. PLoS One 11:e0167869

Xu L, Sun H, Zhang M, Jiang Y, Zhang C, Zhou J, Ding L, Hu Y, Yan G (2017) MicroRNA-145 protects follicular granulosa cells against oxidative stress-induced apoptosis by targeting Kruppel-like factor 4. Mol Cell Endocrinol 452:138-147

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275:1129-1132

Yin Y, Wang S, Sun Y, Matt Y, Colburn NH, Shu Y, Han X (2009) JNK/AP-1 pathway is involved in tumor necrosis factor-alpha induced expression of vascular endothelial growth factor in MCF7 cells. Biomed Pharmacother 63:429-435

Zhang JQ, Gao BW, Wang J, Ren QL, Chen JF, Ma Q, Zhang ZJ, Xing BS (2016) Critical role of FoxO1 in granulosa cell apoptosis caused by oxidative stress and protective effects of grape seed procyanidin B2. Oxid Med Cell Longev 2016:6147345

Zhang QH, Dou HT, Tang YJ, Su S, Liu PS (2015) Lentivirus-mediated knockdown of Kruppel-like factor 9 inhibits the growth of ovarian cancer. Arch Gynecol Obstet 291:377-382

Zhang X, Zhang L, Yang H, Huang X, Otu H, Libermann TA, DeWolf WC, Khosravi-Far R, Olumi AF (2007) c-Fos as a proapoptotic agent in TRAIL-induced apoptosis in prostate cancer cells. Cancer Res 67:9425-9434

Zhao Q, Mao A, Yan J, Sun C, Di C, Zhou X, Li H, Guo R, Zhang H (2016) Downregulation of Nrf2 promotes radiation-induced apoptosis through Nrf2 mediated Notch signaling in non-small cell lung cancer cells. Int J Oncol 48:765-773

Zucker SN, Fink EE, Bagati A, Mannava S, Bianchi-Smiraglia A, Bogner PN, Wawrzyniak JA, Foley C, Leonova KI, Grimm MJ, Moparthy K, Ionov Y, Wang J, Liu S, Sexton S, Kandel ES, Bakin AV, Zhang Y, Kaminski N, Segal BH,

Nikiforov MA (2014) Nrf2 amplifies oxidative stress via induction of Klf9. Mol Cell 53:916-928

Table 2.1. The primers list used in quantitative real-time PCR

Gene	Accession number	Primer sequences[*]	Product size (bp)
ACTB	NM_173979	F: 5´-TGTCCACCTTCCAGCAGAT-3´ R: 5´-TCACCTTCACCGTTCCAGT-3´	249
GAPDH	NM_001034034	F: 5´-CCCAGAATATCATCCCTGCT-3´ R: 5´-CTGCTTCACCACCTTCTTGA-3´	185
NRF2	NM_001011678	F: 5´-CCCAGTCTTCACTGCTCCTC-3´ R: 5´-TCAGCCAGCTTGTCATTTTG-3´	165
KLF4	NM_001105385.1	F: 5´-CAAACCAAAGAGGGGAAGAC-3´ R: 5´-AAACTTCCACCCACAACCAT-3´	175
KLF6	NM_001035271.4	F: 5´-AATCTGAGCTCCTCGGTCAC-3´ R: 5´-TTCCTGCAGCCATTAAAGTG-3´	212
KLF9	NM_001193214.1	F: 5´-TGGCTGTGGGAAAGTCTATG-3´ R: 5´-GAACTGCTTCTCCCCTGTGT-3´	166
c-FOS	NM_182786.2	F: 5´-TGAAAACCATGACAGGAGG-3´ R: 5´-GCAAAGCCGACTTCTCATCT-3´	204
FOSB	NM_001102248.1	F: 5´-ATGACATGCCAGGAACCAGT-3´ R: 5´-TGCTGCCAGTTTGTTTCGTT-3´	218
IRF7	NM_001105040.1	F: 5´-CTGGACTTGAGCATCCTGTAT-3´ R: 5´-ACCTCCCAGTAGACTTTGCA-3´	275
NOTCH1	XM_024999642.1	F: 5´-AGCAACATTAACGAGTGCCA-3´ R: 5´-TCCAGGTTGATCTCGCAGTT-3´	125
E2F1	NM_001206079.1	F: 5´-TCTTGGAATTACTGAGCCGC-3´ R: 5´-CTGGGTCAATCCTTCAAGCC-3´	209
E2F4	NM_001075873.1	F: 5´-TTCATCTGGACCCAATCCTT-3´ R: 5´-GGTGCAAACACTTCTGAGGA-3´	162
SREBF1	NM_001113302.1	F 5-ACCGCTCTTCCATCAATGAC-3´ R 5-TTCAGCGATTTGCTTTTGTG-3´	190
SREBF2	NM_001205600.2	F: 5´-TTCCTCTGCCTCTCCTTTAAC-3´ R: 5´-CACGATCATGCCATTCACCA-3´	183
CYP19A1	NM_174305.1	F: 5´-TGGCTGTGCAGAAAGTATGAA-3´ R: 5´-CAGTGGCGAAATCTATGCTGT-3´	127
CYP11A1	NM_176644	F: 5´-CGGAAAGTTTGTAGGGGACA-3´ R: 5´-ACGTTGAGCAGAGGGACACT-3´	177
STAR	NM_174189	F: 5´-AAATCCCTTTCCAAGGTCTG-3´ R: 5´-ACCAGCATTTCTGCTACTGC-3´	204
PCNA	NM_001034494	F: 5´-CACCAGCATGTCCAAAATAC-3´ R: 5´-CTGAGATCTCGGCATATACG-3´	192
BAX	NM_173894.1	F: 5´-CTCTCCTACTTTGGGACACC-3´ R: 5´-TCCGAAAGAGGTTTATTGTG-3´	259
FOXO1	XM_025000053.1	F: 5´-AAGAGCGTGCCCTACTTCAA-3´ R: 5´-CTCTTCTCCTGGGGGATTTC-3´	187
EGFR	XM_592211	F: 5´-GACCCGAAAGAACTGGACAT-3´ R: 5´-TGTTATATCCAGGCCGACAA-3´	177
INHBA	NM_174363	F: 5´-GCAAGGTCAACATCTGCTGTA-3´ R: 5´-TACAACATGGACATGGGTCTC-3´	262
CASP3	NM_201107726	F: 5´- TGCCACTGTATCAGGGAACA-3´ R: 5´- TGCTCAGCACAAACATCACA-3	192
BCL2L1	XM_015474117.1	F: 5´-AAGTTTTCTGACCCTTTTCC-3´ R: 5´-GGTCCTGGTCCTTATTTCTT-3´	195
AMPKa1	NM_001109802.2	F: 5´-AGCCCTTCCTTCTCTTGCTC-3´ R: 5´-AGGATGCCTGAAAAGCTTGA-3´	246

AMPKa2	NM_001205605.1	F: 5´-AGCAGAGGTCTGGTTCCTCA-3´	155
		R: 5´-TGAGACAGAGGACGACATGC-3´	
Calpain1	NM_174259.2	F: 5´-TGGTCAACCTCATGGATCGC-3´	162
		R: 5´-CAAACTCAATGGCCATCCGC-3´	
Grp78	NM_001075148.1	F: 5´-AACACAGTCTTCGACGCCAA-3´	141
		R: 5´-TTGCCCACCTCCAACATCAA-3´	
Grp94	NM_174700.2	F: 5´-TGCTGTGTGGAGAGGGAATG-3´	129
		R: 5´-TCCTGTGACCACAATCCCAA-3´	
CCNB1	NM_001045872.1	F: 5´-GATACTCCCTCTCCAAGCCC-3´	196
		R: 5´-TGACTGCTTGCTCTTCCTCA-3´	
CCND1	NM_001046273.2	F: 5´-CGACTTCATCGAACACATCC-3´	279
		R: 5´-ATCTTTGCCAGGAGATCCAC-3´	

*F: Forward, R: Reverse
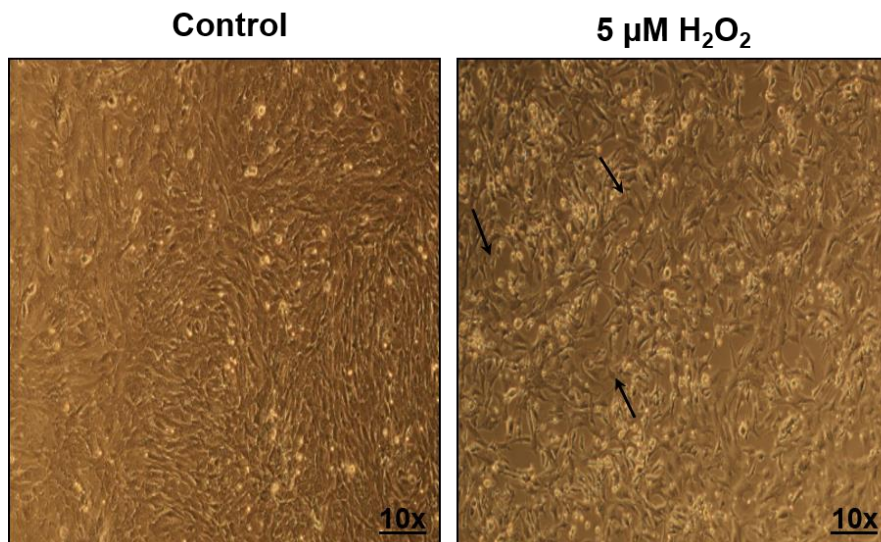
Figure 2.1. Morphology of granulosa cells (10x) in untreated and treated with H_2O_2-induced oxidative stress groups
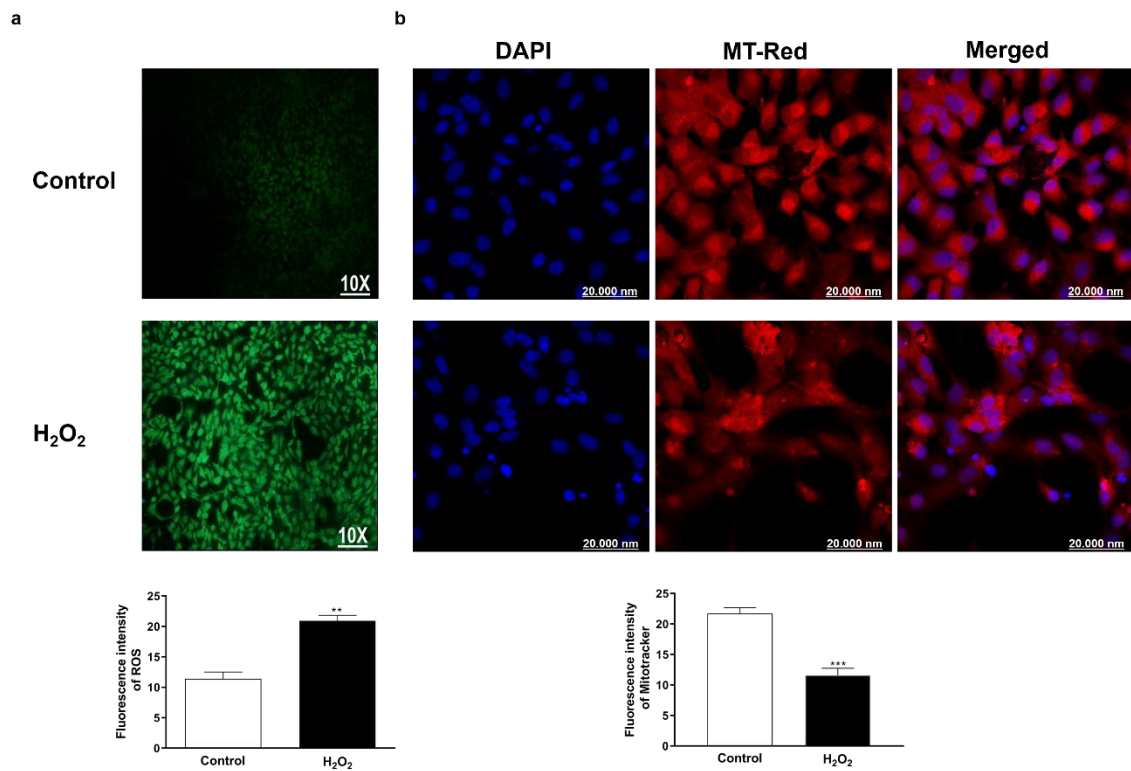
Figure 2.2. Intracellular reactive oxygen species (ROS) accumulation (a) and mitochondrial activity (b) of bovine granulosa cells under oxidative stress conditions: Green color indicates the fluorescence of the 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). The red color indicates mitochondrial activity and the blue color indicates the nuclear staining using 40,6-diamidino-2-phenylindole (DAPI). The fluorescence intensity was measured by ImageJ software. Data are shown as means $\pm$ SEM of three independent replicates (***p≤0.01, ***p≤0.001*)
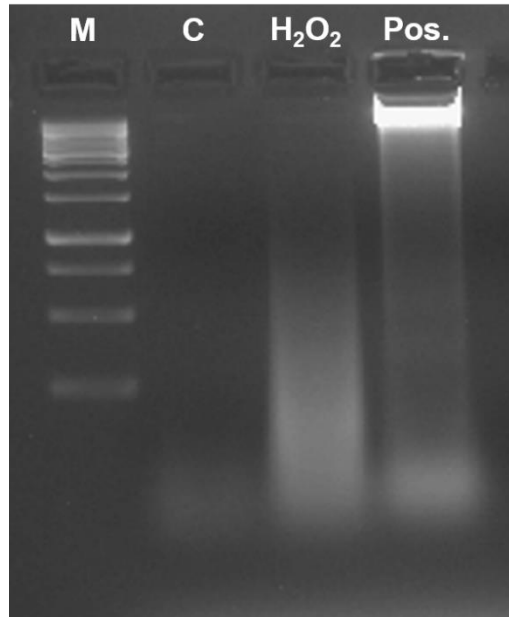
Figure 2.3. Gel electrophoresis of fragmented DNA in bovine follicular granulosa cells exposed to H_2O_2-induced oxidative stress. M indicates a DNA ladder 1kb, C indicates the control group and H_2O_2 shows the cells treated with 5 µM H_2O_2 for 40 min and Pos stands for bovine DNA as a positive control
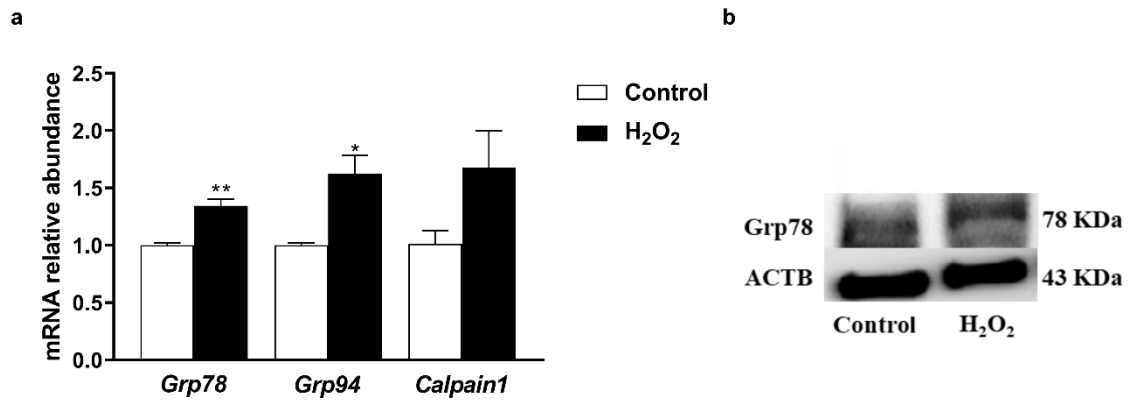
Figure 2.4. The relative mRNA expression profile of endoplasmic reticulum stress marker genes (a) and protein level of Grp78 (b) in bovine granulosa cells exposed to oxidative stress. Data are represented as means ± SEM from triplicates (* *p≤0.05, **p≤0.01*)
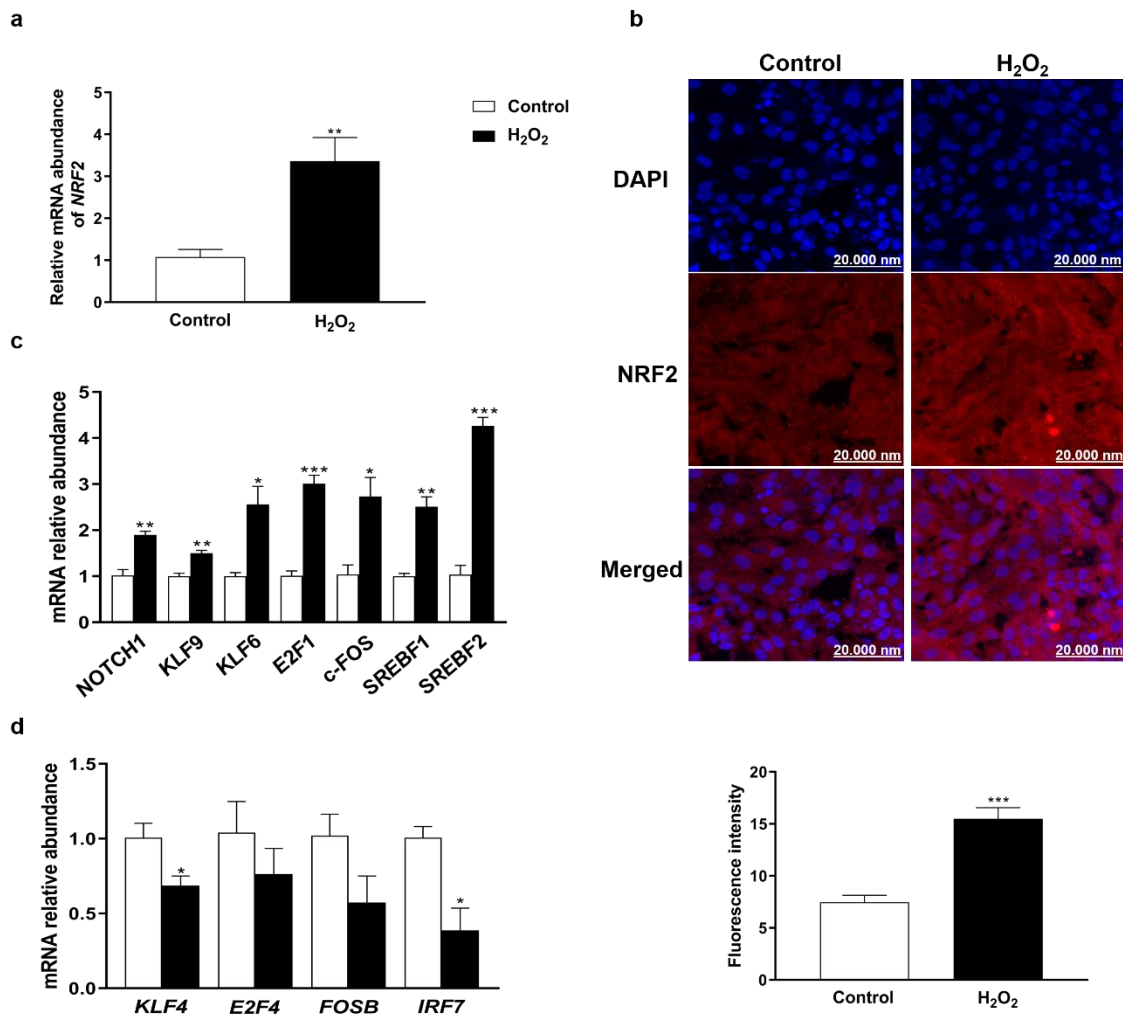
Figure 2.5. Relative abundance of the transcription factors in bovine granulosa cells exposed to oxidative stress. The expression pattern of NRF2 mRNA (a) and its protein (b) in response to oxidative stress, whereby red color indicates the NRF2 protein and the blue color indicates the nuclear staining using 40,6-diamidino-2-phenylindole (DAPI); the effect of oxidative stress on candidate transcription factors, which showed up (c) and downregulation (d). The transcript expression data were normalized to internal ACTB and GAPDH as well as relatively compared to the control group. Data are represented as means ± SEM of triplicates (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). The ImageJ software was utilized to calculate the fluorescence intensity of NRF2 protein
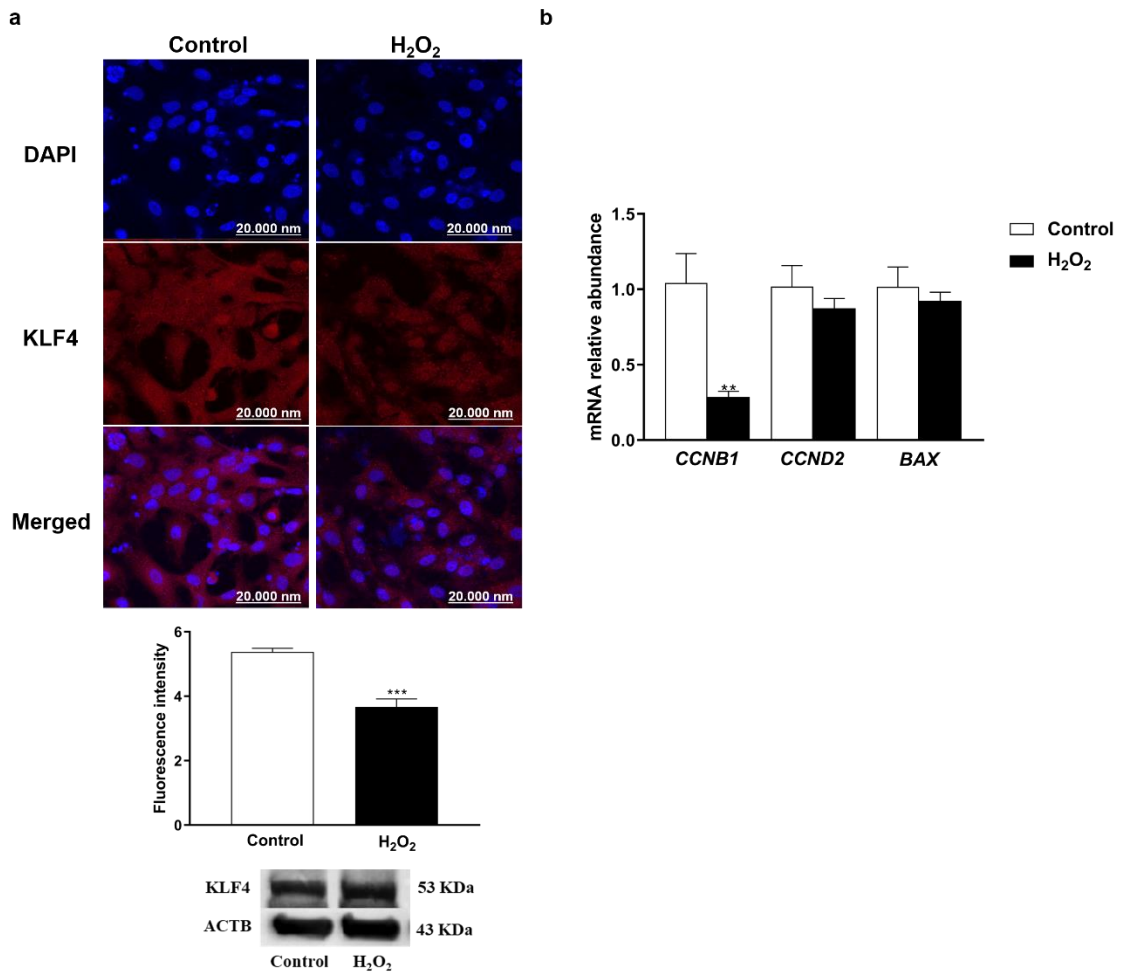
Figure 2.6. Impact of oxidative stress on the protein level of KLF4 (a) and its target genes (b). The red color in immunocytochemistry indicates KLF4 protein and the blue color indicates the nuclear staining using 40,6-diamidino-2-phenylindole (DAPI). The mRNA data were normalized to ACTB and GAPDH as well as relatively compared to the control group. Data represent means ± SEM from triplicates (***p≤0.01, ***p≤0.001*). The Western blot protein analysis of KLF4 was compared to the internal expression of ACTB. The ImageJ software was utilized to calculate the fluorescence intensity of KLF4 protein

a

b

c
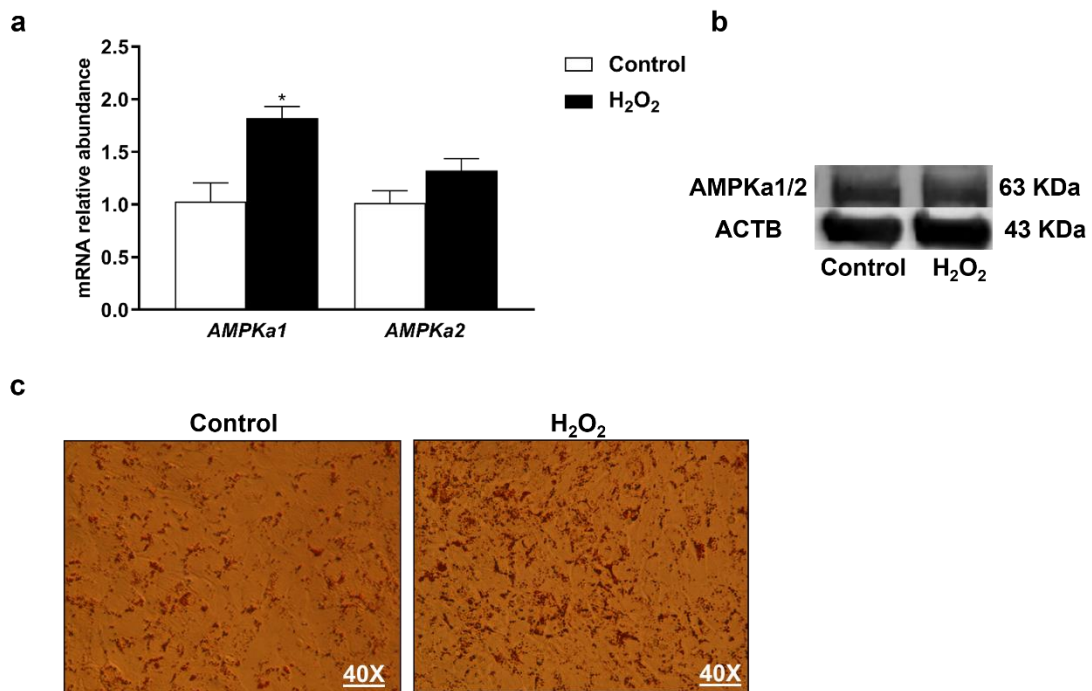
Figure 2.7. The effect of oxidative stress on mRNA expression of AMPKa1 and AMPKa2 (a), the protein level of AMPKa1/2 (b) and lipid accumulation (**c**) in bovine granulosa cells. Data represent means ± SEM of triplicates (*$p \leq 0.05$*)

a

b
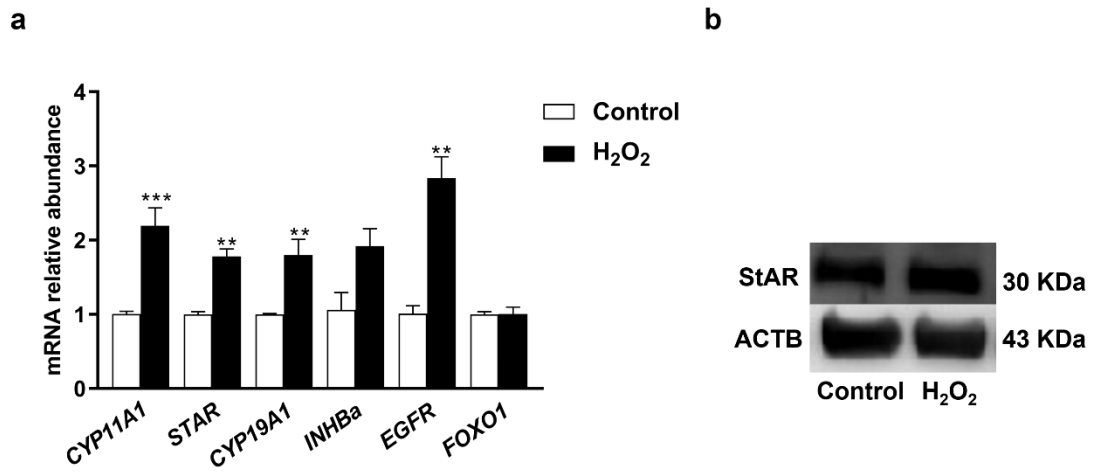
Figure 2.8. The mRNA expression pattern of differentiation-related genes (a) and StAR protein (b) in bovine granulosa cells exposed to H_2O_2-induced oxidative stress. Data are represented as means ± SEM of triplicates (**$**p \leq 0.01$, ***$***p \leq 0.001$)
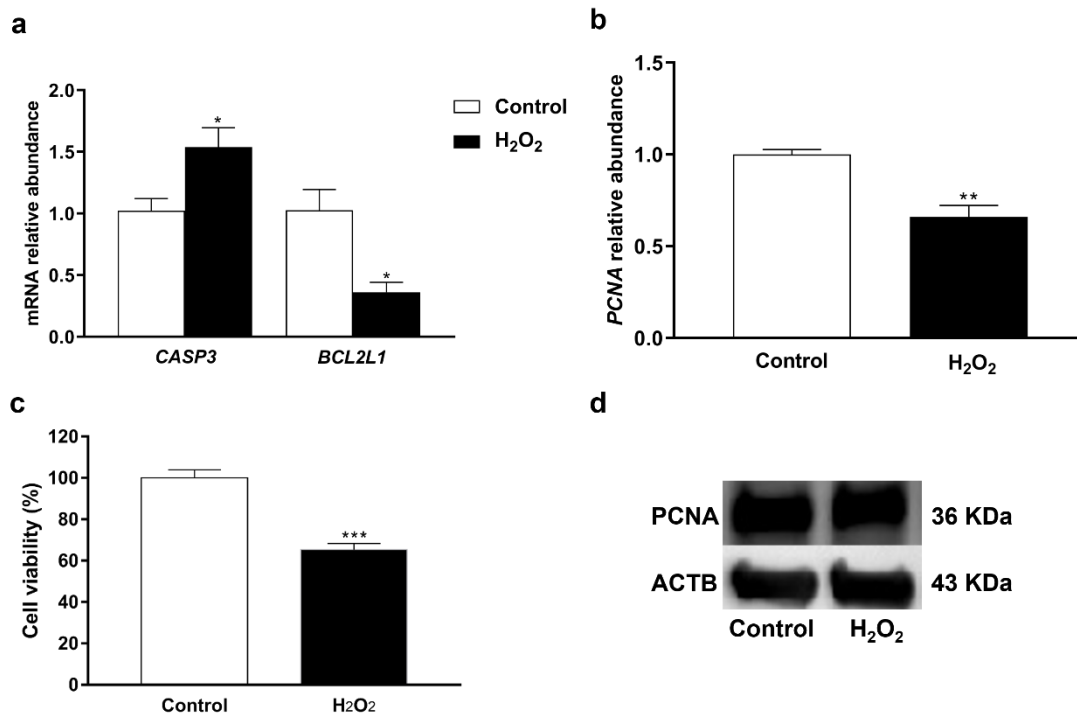
Figure 2.9. Expression pattern of anti-apoptotic, pro-apoptotic (a), proliferation marker (b) genes, cell viability (c) and PCNA protein level (d) in bovine granulosa cells treated with H_2O_2 or untreated control. The geometric mean of ACTB and GAPDH was used for mRNA normalization and the data were relatively compared to the control group. Data are represented as means ± SEM of triplicates (* $p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). The level of ACTB protein was used as an internal control in protein analysis
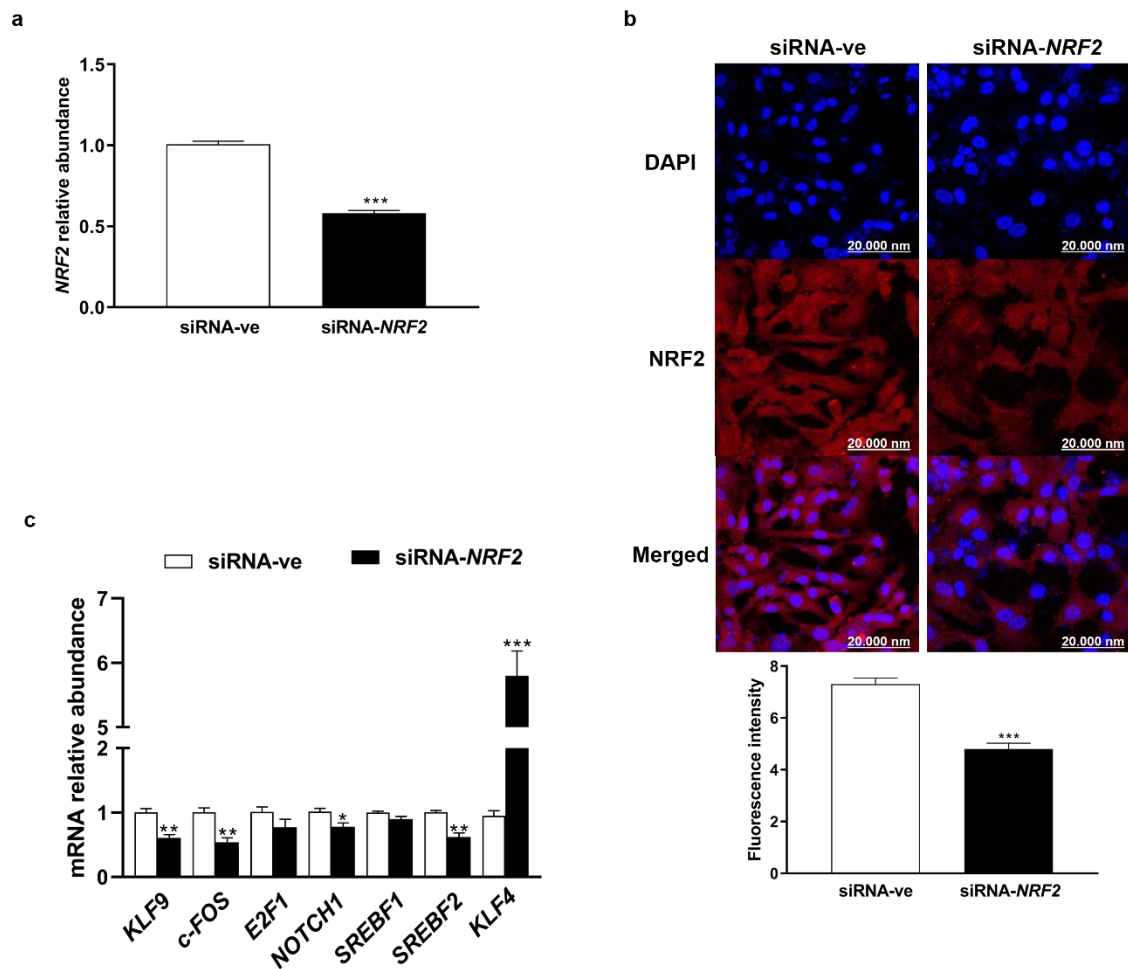
Figure 2.10. Selective Knockdown of NRF2 in cultured bovine granulosa cells altered the expression of candidate transcription factors; the efficiency of utilizing small interference (siRNA) on the mRNA (a) and protein (b) levels of *NRF2*; the impact of siRNA-mediated NRF2 Knockdown on the expression pattern of candidate transcription factors (c). The mRNA data were normalized to internal control of ACTB and GAPDH as well as the data were relatively compared to the negative control group (siRNA-ve). Data are represented as means ± SEM of triplicates (* *p≤0.05*, **p≤0.01*, ***p≤0.001*). The ImageJ software was used to measure the fluorescence intensity of NRF2 protein
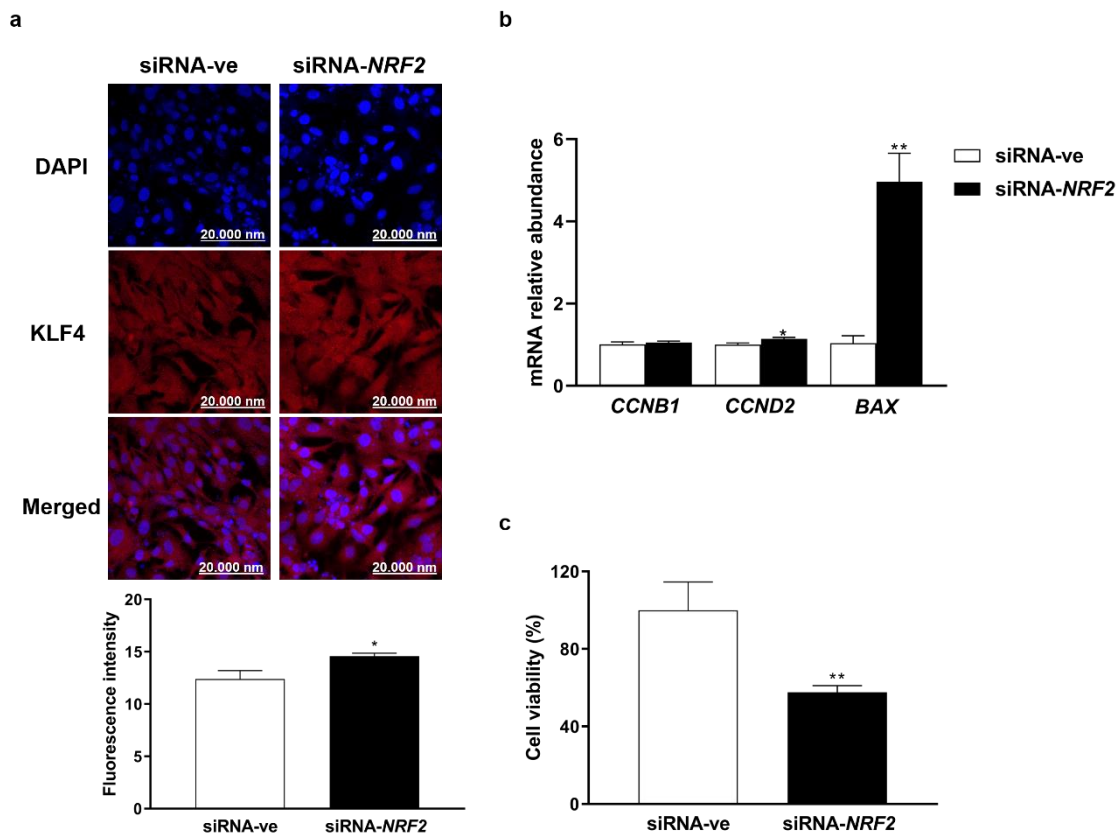
Figure 2.11. The effect of transfection with small interference RNA targeting NRF2 (siRNA-NRF2) on the protein level of KLF4 (a) and its target genes (b) as well as cell proliferation rate (c). Red color indicates the KLF4 protein and the blue color indicates the nuclear staining using 40,6-diamidino-2-phenylindole (DAPI). Data are represented as means ± SEM of triplicates (* $p \leq 0.05$, ** $p \leq 0.01$). The ImageJ software was used to measure the fluorescence intensity of KLF4 protein
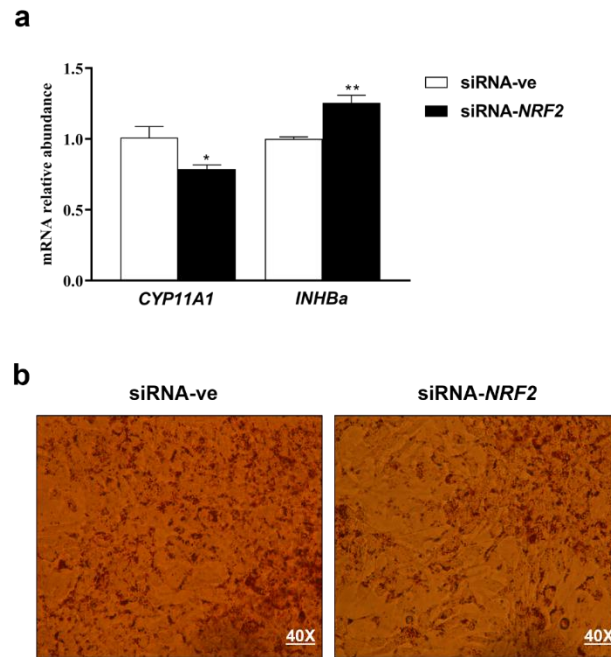
Figure 2.12. The effect of siRNA-mediated NRF2 knockdown (a) and lipid accumulation (b). The red color indicates the oil red O stain. The expression level of ACTB and GAPDH was used to normalize mRNA transcript and the data were relatively compared to the negative control group (siRNA-ve). Data represented as means ± SEM of triplicates (* $p \leq 0.05$, ** $p \leq 0.01$)

Chapter 3: Transcriptional sexual dimorphism in response to oxidative stress

Sexual dimorphic expression and release of transcription factors in bovine embryos exposed to oxidative stress

Mohamed Omar Taqi[1], Mohammed Saeed-Zidane[1], Samuel Gebremedhn[1], Dessie Salilew-Wondim[1], Omar Khadrawy[1], Franca Rings[2,3], Christiane Neuhoff[1], Michael Hoelker[1,2,3], Karl Schellander[1,3], Dawit Tesfaye[1,3*]

[1]Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn
[2]Teaching and Research Station Frankenforst, University of Bonn, Königswinter, Germany
[3]Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

*Correspondence to:
Institute of Animal Science
Animal Breeding and Husbandry Group
Endenicher Allee 15,
Bonn 53115, Germany
E-mail: tesfaye@itw.uni-bonn.de

Short title: Sexually dimorphic response of preimplantation embryos to oxidative stress

3.1 Abstract

Sexually dimorphic differences in genome activity, which is orchestrated by transcription factors (TFs), could explain the differential response of male and female embryos to environmental stressors. To proof this hypothesis, the expression of cellular and extracellular TFs was investigated in male and female bovine embryos *in vitro* cultured either under low (5%) or high (20%) oxygen levels. The intracellular reactive oxygen species (ROS), total cell number, expression of NRF2, KLF4, NOTCH1, E2F1 and SREBF2 along with extracellular vesicles (EVs) biogenesis genes were assessed at the blastocyst stage and their released-EVs. Low blastocyst rate in both sexes due to oxidative stress (OS) was accompanied by increased ROS accumulation and reduced cell number in female embryos. The mRNA and protein levels of NRF2 as well as KLF4 expression were higher in male embryos exposed to OS compared to female embryos. However, the expression of NOTCH1 and E2F1 was higher in female embryos cultured in a high oxygen level. Male embryos exposed to OS released more EVs enriched with NRF2, SOD1 and NOTCH1 accompanied by elevated expression of EVs biogenesis genes. Accordingly, differential expression of TFs and their release into spent media could partially explain sexual dimorphic response of bovine embryos to environmental stresses.

Keywords: Oxidative stress, Sexual dimorphism, EVs, Transcription factors

3.2 Introduction

Early embryogenesis involves various morphological, cellular, and biochemical changes related to genome activity (Stanton et al. 2003), which could be orchestrated by several transcription factors (TFs). TFs are involved in several biological functions such as embryo development, apoptosis, differentiation and stress response (Leite et al. 2017). The exposure of embryos to the *in vitro* culture conditions is known to alter an array of TFs involved in cell death, lipid metabolism, cellular growth, proliferation and oxidative stress response (Gad et al. 2012; Leite et al. 2017). Oxidative stress is triggered by the imbalance between reactive oxygen species (ROS) and its scavenger of antioxidant molecules (Martindale and Holbrook 2002), due to culture media, temperature, pH and oxygen tension (Cagnone and Sirard 2016; Wale and Gardner 2016). For instance, the oxygen levels in oviduct and uterus of most mammalian species (1.5-8.7%) are less than half of atmospheric oxygen level (20%) (Fischer and Bavister 1993), which is widely used in IVF laboratories and consequently lead to elevation of ROS (Amin et al. 2014; Leite et al. 2017; Yoon et al. 2014). Excess accumulation of ROS damages cellular bioactive molecules such as DNA, protein and lipid (Martindale and Holbrook 2002), subsequently compromising the mitochondrial integrity and activity (Amin et al. 2014; Kadenbach et al. 2004) as well as cell number (Amin et al. 2014; Kelley and Gardner 2016; Leite et al. 2017), resulting in abnormal embryonic development or apoptosis (Amin et al. 2014; Kadenbach et al. 2004; Leite et al. 2017).

The molecular defense mechanisms involve nuclear factor (erythroid-derived 2) factor 2 (NRF2 or NFE2L2) signaling pathway and the antioxidants machinery, which is reported to be vital for the survival of embryos under oxidative stress conditions (Amin et al. 2014; Leite et al. 2017). The NRF2 as TF can bind and regulate other TFs such as sterol regulatory element-binding transcription factors (SREBFs) (Amin et al. 2014; Kamisako et al. 2014), NOTCH1 (Zhao et al. 2016) and pluripotency related genes (Jang et al. 2014). The sex ratio is believed to be affected by numerous factors either *in vivo* or *in vitro* including ovarian factors, maternal diets, and culture conditions (Gutiérrez-Adán et al. 2001; Hylan et al. 2009; Iwata 2012; Rosenfeld and Roberts 2004). Furthermore, male and female embryos are known to differ in their transcription (Bermejo-Alvarez et al. 2010b), epigenetic profile (Bermejo-Alvarez et al. 2008) and metabolite consumption (Gómez et al. 2018), which may be associated with their differential response to environmental insults. So far, this sexual dimorphic response to stress between male and

female embryos is controversial and not fully understood. Some argue that female embryos are more tolerant to heat stress than male embryos (Pérez-Cerezales et al. 2005). This could be attributed to the transcriptional dimorphism derived from unaccomplished X-chromosome inactivation (XCI) during early embryo development (Bermejo-Alvarez et al. 2010b). This phenomenon is not only correlated with the upregulation of several genes linked to sex chromosomes but also to autosomal chromosomes (Pérez-Cerezales et al. 2018) like Glucose-6-phosphate Dehydrogenase (G6PD), which is an X-linked gene related to oxygen-free radicals. In bovine, the G6PD has been found to be upregulated in female morula than male counterparts exposed to oxidative stress accompanied by enhanced developmental componence (Iwata et al. 2002). Contrastingly, others reported that the bovine female embryos were more susceptible to apoptosis than male counterparts (Ghys et al. 2016; Oliveira et al. 2016) following fertilization either with sex-sorted or unsorted semen and cultured under two different culture conditions (Ghys et al. 2016).

Extracellular vesicles (EVs) are small vesicles (30 nm to 5 µm) originated in cell lumen of most cells and secreted into the extracellular space (Anderson et al. 2010; Chiang and Chen 2019; Lawson et al. 2016; Ståhl et al. 2019) including biological fluids such as plasma, milk, saliva, semen, urine (Lawson et al. 2016), follicular fluid (Hung et al. 2015) and uterine aspirates (Campoy et al. 2016). Based on their size, EVs can be subdivided into apoptotic bodies (1000-5000 nm), microvesicles (100-1000 nm) and exosomes (30-200 nm) (de la Torre Gomez et al. 2018; Lawson et al. 2016; Sun et al. 2010). They carry a cargo of bioactive molecules such as DNA, RNAs, proteins to be transferred into the extracellular environment in order to maintain cellular homeostasis (Takahashi et al. 2017) and/or transfer genetic material to the neighbor or distant recipient cells (Saeed-Zidane et al. 2017). Additionally, its cargo content varies depending on the cell origin (de la Torre Gomez et al. 2018), physiological status (Hung et al. 2017; Navakanitworakul et al. 2016), inflammation (Jolly et al. 1975) and stress status (Beninson et al. 2014; Bewicke-Copley et al. 2017) of the donor cells. However, the effect and regulation of EVs biogenesis and secretion in bovine embryos during oxidative stress condition is not yet fully understood. Hence, the current study was designed to understand the cellular and extracellular defense mechanisms of male and female bovine embryos exposed to oxidative stress under *in vitro* culture condition.

3.3 Materials and methods

3.3.1   Oocyte collection and maturation

Ovaries were obtained from a nearby slaughterhouse and transported to the laboratory in 0.9% physiological saline solution (NaCl) within 2-3 h. Upon arrival, the ovaries were rinsed with prewarmed (37 °C) 70% ethanol followed by 2-3 times washing in fresh phosphate buffer saline (PBS), and cumulus-oocyte complexes (COCs) were then aspirated from small follicles (2-8 mm), using sterilized syringe attached to 18 G needle. Oocytes with homogenous cytoplasm and surrounded by multiple layers of compacted cumulus cells were then selected for *in vitro* maturation. Groups of 50 COCs were transferred to TCM 199 modified culture media (Sigma-Aldrich, Munich, Germany) supplemented with 4.4 mM HEPES, 33.9 mM NaCHO3, 2 mM pyruvate, 2.9 mM calcium lactate, 55 mg/mL gentamicin and 12% (v/v) heat-inactivated oestrus cow serum (OCS) (Schellander et al. 1990) and then cultured for 22-24 h at 39 °C under 5% CO_2 in air with maximum humidity.

3.3.2   *In vitro* embryo production

Following maturation, COCs were co-incubated for 20 h under 20% oxygen level with 2×10^6 sperms/mL sex-sorted sperm in F-TALP medium supplemented with 20 mM penicillamine, 10 mM hypotaurine, 2 mM noradrenaline, 6 mg/mL BSA, 50 mg/mL gentamicin and 1 mg/mL heparin (Parrish et al. 1986). Subsequently, presumptive zygotes were denuded by repeated pipetting and transferred to 400 µL of SOF culture medium (Holm et al. 1999) supplemented with 10% exosomes-depleted fetal bovine serum (FBS) in four-well dishes (Thermo Fisher Scientific, Roskilde; Denmark). Presumptive zygotes were incubated in two different oxygen levels 5% CO_2 in air (20% supraphysiological level) or 5% CO_2, 5% O_2 and 90% N_2 (5% physiological level) until the blastocyst stage (day 8). After recording developmental data, the male and female embryos were collected and washed twice in PBS and then snap-frozen in cryotubes containing lysis buffer [40 U mL^{-1} RNasin (Promega, WI, USA), 0.8% Igepal (Sigma-Aldrich, MO, USA), 5 mM dithiothreitol (DTT) (Promega, WI, USA)]. The validation of the sex of IVP embryos (10 blastocysts per each replicate) using sex-sorted semen was performed using multiplex polymerase chain reaction (PCR) as described before (Rattanasuk et al. 2011). Briefly, a combination of two primers, bovine specific primer (BSP,   Forward:5'-TTTACCTTAGAACAAACCGAGGCAC-3'   and   Reverse:5'-

TACGGAAAGGAAAGATGACCTGACC-3' with amplicon size 538bp) and Y-specific primer (BY, Forward:5'-CTCAGCAAAGCACACCAGAC-3' and Reverse:5'-GAACTTTCAAGCAGCTGAGGC-3' with amplicon size 300bp), was used to determine the sex of embryos. A total of 20 µL amplification reaction for each replicate was subjected to thermal cycle program of initial preheating with 95 °C for 2 min, followed by 35 cycles of 20 sec at 95 °C, 45 sec at 52 °C and 50 sec at 72 °C followed by final extension for 10 min at 72 °C. Finally, PCR products were electrophoresed on 2% agarose gel and the image was visualized under ultraviolet light using ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany).

3.3.3   Intracellular ROS detection

To investigate the effect of oxidative stress (20% O_2) on intracellular ROS accumulation in male and female bovine embryos, the ROS level in blastocyst was detected using the 6-carboxy- 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescent probe, (Life Technologies, Darmstadt, Germany). Fifteen blastocysts from each group were incubated with 400 µL of 5 µM H2DCFDA for 20 min in dark at 37 °C. Thereafter, the embryos were washed twice in PBS-PVA and the images were immediately acquired under an inverted microscope (Leica DM IRB, Germany), using a green fluorescence filter. The fluorescent intensity was analyzed as a single embryo for each group using ImageJ 1.48v software (National Institutes of Health, USA, http://imagej.nih.gov).

3.3.4   Total cell count

Total blastocyst cell number of male and female bovine embryo cultured under low (5%) or high (20%) oxygen levels were quantified using nuclear fluorescence staining with the glycerol-based Hoechst 33342 (Sigma-Aldrich, Munich, Germany) according to the manufacturer's procedure. For that, ten blastocysts from each group were fixed for 5 min in a solution containing 2% formalin and 0.25% glutaraldehyde. The fixed blastocysts were mounted and stained for 10 min with glycerol-based Hoechst 33342 (12.5 mg/mL) solution on clean glass slides. The stained nuclei were visualized using a fluorescent microscope (Olympus, Tokyo, Japan) with a blue filter (excitation: 330-385 nm; emission: 420 nm; dichromatic: 400 nm). The cell number was recorded for individual blastocysts from each cultured group using Zen 2.3 V (blue edition) (https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html).

3.3.5   RNA extraction and cDNA synthesis

Four biological replicates (pool of ten blastocysts/each) embryos from each group were used for RNA extraction. Total RNA was extracted using a PicoPure RNA isolation kit (Arcturus, Munich, Germany) according to the manufacturer's instruction. On-column DNA digestion was performed using RNase-free DNase enzyme (Qiagen GmbH, Hilden, Germany). After two washes with washing buffer, the RNA was eluted in 12 µL elution buffer and stored at -80 °C until further use. Additionally, the total RNA from EVs of four biological replicates was isolated using Norgen's exosomal RNA isolation kit (Norgen biotek, Canada) following the manufacture's procedure. Briefly, a combination of lysis buffer was added to certain volume of PBS-CMF (calcium-magnesium free) containing EVs followed by vortex and incubation for 10 min at room temperature, respectively. After incubation, 500 µL of absolute ethanol was added to the mixture and mixed well by vortexing. Appropriate volume from mixture was transferred to mini spin column and then centrifuged at 3300 g. After withdrawal of the flowthrough, the mini spin column was washed twice with washing buffer each time followed by centrifugation at 3300 g for 30 sec. Afterwards, the mini spin column was dried with centrifugation at 13000 g for 1 min at room temperature. Finally, the RNA was eluted in appropriate amount of elution buffer and then stored at -80 °C till further application.

The RNA concentration was measured (Supplementary table 3.1 and 2) using 8000 NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Germany) and equal quantities of RNA input were used for cDNA synthesis. The cDNA synthesis was performed using First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Germany). Briefly, an equal amount of RNA was incubated with 0.5 µL of Oligo-dT and 0.5 µL Random Primer, followed by incubation at 65 °C for 5 min. Besides this, a master mix containing 4 µL of 5x reaction buffer, 1 µL of RiboLock RNase inhibitor, 2 µL of 10 mM dNTP mix and 2 µL of MMuLV reverse transcripts were prepared and gently mixed by pipetting. At the end of incubation, a total of 9 µL was added to each specimen, and then incubated at 25 °C for 5 min, then 37 °C for 60 min followed by 70 °C for 5 min to terminate the reaction. After the incubation time, the cDNA was kept at -20 °C for gene expression analysis.

3.3.6   Quantitative Real-Time PCR

The transcript level of developmental and stress-related TFs namely, nuclear factor (erythroid-derived 2) factor 2 (NRF2), Krüppel-like factor 4 (KLF4), notch receptor 1 (NOTCH1), sterol regulatory element-binding transcription factor 2 (SREBF2), and E2F transcription factor 1 (E2F1), TFs target genes namely; catalase (CAT), superoxide dismutase 1 (SOD1), POU class 5 homeobox 1 (POU5F1), and GATA binding protein 4 (GATA4) as well as genes associated with EVs biogenesis and secretion namely; programmed cell death 6 interacting protein (PDCD6IP/ALIX) , vacuolar protein sorting 4 homolog B (VPS4B), STEAP3 metalloreductase (TSAP6), RAB11 family interacting protein 2 (RAB11FIP1), RAB35, member RAS oncogene family (RAB35), and RAB27A, member RAS oncogene family (RAB27A) was investigated in male and female bovine embryos cultured under 5 and 20% oxygen level using iTaq™ primaQUANT real-time PCR (qRT-PCR) Master Mixes (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany) in Applied Biosystem® StepOnePlus™ (Applied Biosystems, Foster City, California, USA). Primers were designed using primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 3.1). The master mix containing cDNA, optimized amount of forward and reverse primers, and SYBR green master mix was run in a thermal cycler program of 95 °C for 3 min initial denaturation, followed by 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C. A melting curve was generated to verify the amplicon specificity. The expression level of ACTB and GAPDH was used for normalization for embryonic transcripts, while 18S was used as a third housekeeping gene for EV-coupled transcript profiling. The relative abundance of gene transcript was expressed as arbitrary unit calculated by $2^{-\Delta Ct} = 2^{-(Ct\ target\ gene - Ct\ housekeeping\ gene)}$ (Livak and Schmittgen 2001).

3.3.7   Immunohistochemistry

To quantify and localize the NRF2 and KLF4 proteins in male and female bovine embryos cultured under 5% and 20% oxygen levels, immunohistochemistry assay was conducted. For this, ten embryos from each group were fixed using 4% paraformaldehyde and kept at 4 °C overnight. Thereafter, the fixed embryos were washed four times for 5 min each with glycine in PBS (GPBS), followed by permeabilization step using 0.5% (v/v) Triton-X100 (Sigma-Aldrich, Munich, Germany) in PBS for 3 h at room temperature. After the incubation time, the samples were incubated with 4% donkey serum for 1 h at room

temperature, followed by incubation at 4 °C overnight with primary rabbit polyclonal antibody for NRF2 (1:100, Biorbyt, UK) and KLF4 (1:100, Thermo Fisher, Germany) dissolved in 4% donkey serum. Following incubation, the embryos were washed four times with PBS-PVA, followed by incubation with Alexa flour goat anti-rabbit secondary antibody (1:350 dilution, Lifespan Biosciences) for 3 h at 37 °C. Afterwards, the specimens were mounted on the slide with vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Finally, samples were visualized under confocal microscopy CLSM LSM-780 and the signals were analyzed with ImageJ 1.48v (National Institutes of Health, USA, http://imagej.nih.gov).

3.3.8 EVs isolation and characterization

Four biological replicates of the total of 350 µL culture media from male and female bovine embryos cultured under 5% and 20% oxygen level were collected for EVs isolation. Briefly, the spent media were subjected to differential centrifugation at 4 °C starting from 300 g, 10 min to remove cells followed by 2000 g, 15 min to remove dead cells; and finally, 10000 g, 30 min to remove cell debris, macroparticles, and apoptotic bodies. Then, the supernatant was collected and subjected to ultracentrifugation at 120,000 g for 70 min at 4 °C in a Beckman SWTi55 rotor in order to isolate EVs. Afterward, the isolated EVs were washed in PBS-CMF and then ultracentrifuged using the aforementioned procedure. Finally, the isolated EVs were suspended in PBS-CMF and kept at -80 °C for further investigation.

The purity of the isolated EVs was examined using immunoblotting for the presence of EV marker CD63 protein and for the absence of mitochondrial cellular marker, Cytochrome c (CYCS). Briefly, an equal amount of EVs solution from each group was incubated with 2x SDS loading buffer at 95 °C for 5 min and then loaded onto 12% SDS-PAGE gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Protran®, Schleicher & Schuell Bioscience) using a semi-dry blotting system (Bio-Rad, Munich, Germany). Subsequently, the membrane was blocked using 1x blocking solution (Carl Roth GmbH) for 1 h at room temperature followed by incubation overnight at 4 °C with the primary rabbit polyclonal antibody against CD63 (1:1000) (System BioSciences, USA) and goat polyclonal against CYCS (1:350) (Santa Cruz Biotechnology Inc, Germany). After the incubation time, the membrane was washed three times with diluted Tris-buffer saline with Tween 20 (TBST) followed by incubation for

1 h at room temperature with goat anti-rabbit (1:5000) (System BioSciences, USA) and donkey anti-goat (1:5000) (Santa Cruz Biotechnology Inc, Germany) secondary antibodies. Afterward, the membrane was washed with diluted TBST followed by incubation with Clarity ECL Substrate (Bio-Rad, Munich, Germany). Finally, the bands were visualized and the images were picked up using the ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany).

The concentration and size of EVs were evaluated using NanoSight NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK). Briefly, a total of 50 µL from isolated EVs were diluted in 1 mL PBS-CMF, and then five consecutive videos were recorded. The recorded videos for each sample were analyzed using NTA software to obtain mean, mode of the particle size and concentration per mL of volume.

3.3.9 Electron microscopy

A total of 30 µL from isolated EVs were used for visualization under electron microscopy. Briefly, the sample was dropped on parafilm and co-incubated with Formvar/carbon-coated grids for 5 min at room temperature. Afterward, the Formvar/carbon-coated gird was washed with PBS and subsequently stained with 2% uranyl acetate followed by washing with PBS. Finally, the grids were dried and the images were acquired using electron microscopy.

3.3.10 Statistical analysis

The data were statistically analyzed using two-way analysis of variance (ANOVA) followed by multiple pair-wise comparisons using the Tukey post hoc test (GraphPad Prism Version 7). The data are presented as mean ± SEM of biological quadruplicate. Statistical significance was determined at $p \leq 0.05$.

3.4 Results

3.4.1 Effect of oxidative stress on cleavage and blastocyst rate of male and female embryos

The culture of male and female embryos under 5% or 20% oxygen levels revealed no significant difference in cleavage rate (Figure 3.1A). However, blastocyst rates at days 7 and 8 were significantly reduced under 20% compared to 5% oxygen level in both male and female embryos (Figure 3.1B and C). Interestingly, the day 8 blastocyst rate of male

embryos was significantly higher compared to female counterparts at both oxygen concentrations. The sex of embryos produced using sexed semen was validated using a combination of primers and results are indicated in supplementary Figure 3.1.

3.4.2 Higher atmospheric oxygen induced ROS accumulation in both male and female embryos

Embryos cultured under 20% oxygen level showed significantly elevated intracellular ROS accumulation irrespective of the sex of embryo (Figure 3.2). However, male embryos exhibited a significantly higher accumulation of ROS when cultured under 5% oxygen level compared to female counterparts. Nonetheless, there were no significant differences in ROS accumulation between male and female embryos cultured under 20% oxygen level.

3.4.3 Oxidative stress resulted in reduced total cell number

Oxidative stress due to high oxygen level reduced the blastocyst cell number in both male and female embryos (Figure 3.3). Moreover, the impact of higher oxygen level was pronounced in female blastocysts, as evidenced by a significant reduction in total cell number compared to male embryos cultured under 20% oxygen level. The blastocyst cell number did not differ between male and female embryos cultured under 5% oxygen level.

3.4.4 Oxygen level altered the expression of TFs in male and female embryos

Analysis of the differential response of male and female bovine embryos in terms of expression of TFs related to oxidative stress response (NRF2), differentiation and apoptosis (KLF4, NOTCH1), cell cycle (E2F1) and cholesterol biosynthesis (SREBF2) was performed at the blastocyst stage. Results showed that the expression pattern of NRF2 was significantly higher in male blastocysts cultured under 20% oxygen level compared to those cultured under 5% oxygen level (Figure 3.4). Similarly, NRF2 tended to be higher in female embryos cultured under 20% compared to 5% oxygen level. Likewise, the transcript abundance of KLF4 was significantly higher in male embryos exposed to higher oxygen tension compared to the low oxygen level. However, no significant difference was observed in the expression level of NOTCH1 in both male and female embryos cultured under 20% oxygen level compared with 5% oxygen level. On the other hand, NOTCH1 was significantly higher in female embryos cultured under 20%

oxygen level compared to their male counterparts cultured under the same oxygen level. The expression of E2F1 did not show any significant difference between male embryos cultured under 5% or 20% oxygen levels. Contrary to this, E2F1 was significantly higher in female blastocysts derived from 20% oxygen level compared to those from 5% or male embryos cultured under 20% oxygen level. Male embryos cultured under both 5% and 20% oxygen level showed elevated SREBF2 expression pattern compared to female blastocysts cultured under both oxygen levels. Interestingly, exposure of male embryos to elevated oxygen level led to significant upregulation of SREBF2 compared to those cultured under lower oxygen level. On the other hand, the female embryos did not show any significant difference after culture at 5% and 20% oxygen levels in terms of SREBF2 expression.

3.4.5 Oxidative stress induced expression of NRF2 and KLF4 proteins

The protein expression of selected TFs in male and female blastocysts cultured under 5% or 20% oxygen levels revealed that NRF2 protein was significantly higher and proportionally localized in the nucleus of both male and female embryos cultured under 20% oxygen level than those cultured under 5% oxygen level. As opposed to the mRNA level, the protein level of NRF2 was higher in male embryos cultured under 20% oxygen than their female counterpart (Figure 3.5), and no significant difference was noticed between male and female embryos cultured under 5% oxygen level.

Even though the KLF4 protein level tended to be higher in embryos cultured under 20% compared to 5% counterparts, the male and female embryo did not show a statistically significant difference in the KLF4 protein abundance irrespective of the oxygen level (Figure 3.6).

3.4.6 Dysregulation of antioxidant and differentiation genes after induced oxidative stress

Exposure of embryos to higher oxygen level significantly increased the expression of CAT and SOD1 in male embryos (Figure 3.7). However, only female embryos showed a significantly higher abundance of CAT in response to high oxygen level, as opposed to SOD1, which was indifferent in female embryos cultured under higher or lower oxygen levels. Similarly, the expression of differentiation-related gene POU5F1 was significantly higher in male and female embryos cultured under 20% oxygen level compared to the 5%

counterparts. However, no significant differences in transcript abundance of CAT, SOD1 and POU5F1 were observed between male and female bovine embryos cultured under the same oxygen level. The expression of GATA4 was not different within the sex of embryo cultured under either 5% or 20% oxygen level. However, under both oxygen levels, male embryos exhibited elevated expression of GATA4 compared to female embryos.

3.4.7 Characterization of EVs derived from male and female embryos spent media

The presence of EVs in the spent culture media of male and female embryos was confirmed using EV marker protein namely CD63 and their purity or absence cellular contamination was checked by the absence of CYCS in the isolated EVs (Figure 3.8A). Subsequently, the concentration and size of particles were determined using nanoparticle tracking analysis (NTA) and the morphology of the isolated EVs was assessed using transmission electron microscopy (TEM) (Figure 3.8B and C). The size distribution of EVs was ranging from 117 ± 2.1 nm to 145.2 ± 1.1 nm irrespective of the embryo sex and oxygen level. The concentration of EVs was higher in male embryos cultured under 20% oxygen level compared to those under 5% oxygen level. Interestingly, the average size of EVs was tended to be higher in male embryos derived from 20% oxygen level than those derived from 5% oxygen level. Interestingly, low concentration of EVs was found in female embryos derived from 20% compared to those derived from 5% oxygen level. However, the EVs released into cultured media of male embryos exposed to oxidative stress had a higher concentration and bigger average size than EVs released from female embryos.

3.4.8 Expression of EV biogenesis and secretion related-genes under oxidative stress condition

To evaluate whether the difference in the concentration of EVs is attributed to the impact of oxidative stress, the expression levels of genes related to EVs biogenesis (ALIX, VPS4B and TSAP6) and secretion (RAB11FIP1, RAB35 and RAB27A) were investigated. Results revealed that the expression levels of ALIX, VPS4B, RAB11FIP1 and RAB27A were significantly higher in male embryos cultured under 20% oxygen level compared to the 5% counterparts (Figure 3.9). However, only the expression of ALIX was higher in female embryos cultured under 20% oxygen level. Exposure of embryos to higher oxygen level led to elevated expression of EV biogenesis related gene VPS4B and

secretion-related genes RAB11FIP1 and RAB27A in male embryos compared to female embryos cultured under the same oxygen level. However, the expression of TSAP6 and RAB35 was not affected by both the sex of embryo and oxygen level.

3.4.9 EV-coupled transcript releases due to oxidative stress

In an attempt to determine whether the changes occurred in the embryos due to oxidative stress could affect the communication between embryos via the release of stress indicators loaded in EVs, the mRNA content of released EVs was isolated and quantified for mRNA level of stress-related candidate TFs. Results revealed that the NRF2 (Figure 3.10) carried by EVs released from male embryos cultured under 20% oxygen level was higher compared to EVs released from male embryos cultured under 5% oxygen level and female embryos cultured under 20% oxygen. Similarly, SOD1 was significantly higher in EVs released from male embryos exposed to oxidative stress compared to those cultured under normal conditions. The transcript level of CAT, KLF4 and E2F1 did not show significant differences among all investigated groups. It is noteworthy that the EVs released from male embryos exposed to oxidative stress showed a significantly higher amount of NOTCH1 transcript than those cultured under 5% oxygen level and the EVs released from female embryos exposed to oxidative stress. EVs released from female embryos under 5% and 20% oxygen levels did not show significant differences in terms of NOTCH1 transcript level.

3.5 Discussion

The suboptimal culture conditions of the *in vitro* production system are partly responsible for the reduction in number and quality of blastocysts (Farin and Farin 1995; Gad et al. 2012). In addition to the culture media, the higher oxygen level in *in vitro* system is responsible for contributing to the suboptimal environment (Amin et al. 2014; Kelley and Gardner 2016; Leite et al. 2017). However, previous evidence indicated that there was no significant difference observed in terms of embryo development between maturation followed by fertilization at high oxygen tension and maturation at low oxygen followed by fertilization at high oxygen level, but a dramatic effect was observed in case of fertilization at low oxygen level (Bermejo-Alvarez et al. 2010a). Accordingly, the maturation and fertilization were done at high oxygen tension in the current study. Furthermore, here we showed that irrespective of the sex of embryo, those embryos

cultured under 5% oxygen level showed higher development rate of blastocyst than those under 20% oxygen level, as it has been reported before (Amin et al. 2014; Leite et al. 2017). Differences in metabolism (Gómez et al. 2018) and genetics (Bermejo-Alvarez et al. 2010b) between male and female bovine embryo could contribute to skewness in sex ratio of the resulted embryos (Oliveira et al. 2016; Xu et al. 1992). Similarly, our results showed that the blastocyst rate at day 7 and 8 (Figure 3.1) were skewed toward the male embryos when cultured under either 5% or 20% oxygen levels. As it was been reported previously (Amin et al. 2014; Leite et al. 2017; Yoon et al. 2014), embryos cultured under higher oxygen tension (20%) revealed elevated intracellular ROS accumulation irrespective of the sex of the embryos (Figure 3.2). However, male embryos exhibited higher ROS accumulation than female embryos at lower oxygen level, which may be attributed to higher metabolism rate of male embryos compared to female counterparts (Tiffin et al. 1991). Consistently, metabolite analysis of the spent culture media of male and female bovine embryos cultured under low oxygen tension revealed extensive metabolite exchange of male embryos than the female counterpart (Gómez et al. 2018). However, this difference was faded out under high oxygen tension, which may be associated with the potential role of glucose in ROS scavenging process (Andrisse et al. 2014).

The higher oxygen tension is also reported to compromise the cell proliferation, as evidenced by subsequent reduction in total cell number of embryos (Amin et al. 2014; Leite et al. 2017; Van Soom et al. 2002; Yoon et al. 2014). Likewise, we found that embryos exposed to high oxygen level had a reduced total cell number (Figure 3.3) independent of the sex of embryo, which could be associated with dysregulation of development and apoptosis-related genes (Leite et al. 2017). The negative effect of high oxygen level in terms of cell number was more pronounced in female embryos than male counterparts. This fact is in agreement with Dallemagne and his colleagues (2018), who showed that exposing bovine embryos to oxidative stress from day 5 to day 7 in presence of FCS but not bovine serum albumin supplemented with insulin, transferrin and selenium (BSA-ITS) resulted in a reduction of total cell number and increasing apoptosis in female embryos compared to male counterparts. Moreover, higher cell number was observed in male embryos than female cultured under 5% oxygen level in presence FCS or BSA-ITS, which was not in agreement with the current study (Dallemagne et al. 2018). Supporting to our results, no significant difference in total cell numbers was observed between male

and female bovine embryos cultured under low oxygen tension (Siqueira and Hansen 2016), which indicates the contribution of culture media in the variation between male and female embryos under either normal or stress conditions.

In fact, one of the cellular mechanisms in response to suboptimal conditions is activation/suppression of transcription to maintain cellular homeostasis. This has been elucidated by the fact that higher intracellular ROS level induces the expression of NRF2 in bovine granulosa cells (Saeed-Zidane et al. 2017) and preimplantation embryos (Amin et al. 2014). NRF2 is a TF found to be sequestrated in the cytoplasm via Kelch-like ECH-associated protein 1 (KEAP1). During stressor-induced NRF2 activation, the NRF2 disengages from KEAP1 and translocated in the nucleus to bind with a specific DNA motif called Antioxidant Response Element (ARE) to induce the antioxidant machinery (Bryan et al. 2013; Zhang 2006). Consistent with this, we found that the mRNA expression (Figure 3.4) and protein pattern (Figure 3.5) of NRF2 were higher in male embryos cultured under 20% oxygen level compared to 5% oxygen counterparts. However, female embryos showed only elevation of their protein. This was further followed by increased expression of antioxidant genes namely CAT and SOD1 (Figure 3.7), which was clearly pronounced in male embryos than the female ones (Figure 3.4A). Besides its role in activation of antioxidant machinery, NRF2 has been implicated in the regulation of other cellular functions such as lipid metabolism (Amin et al. 2014; Kamisako et al. 2014), via regulation of SREBFs genes. The SREBFs family has three subtypes namely SREBF1a, SREBF1c and SREBF2, which are involved in the regulation of cellular fatty acids and cholesterol biosynthesis through binding to Sterol Regulatory Element (SRE) (Daemen et al. 2013; Shimano 2001). Our results revealed that the expression of SREBF2 was increased under high oxygen tension (Figure 3.4), which may be occurred in response to endoplasmic reticulum stress-induced cholesterol accumulation via increasing SREBP2 cleavage (Colgan et al. 2007). Interestingly, the expression of SREBF2 was significantly higher in male compared to female counterparts either cultured in 5% or 20% oxygen levels, which may be attributed to developmental dimorphism in favor of male embryos (Xu et al. 1992). Recent studies also evidenced that bovine male embryos release higher lipids and lipid-like molecules into culture media than female counterparts (Gómez et al. 2018).

NOTCH1 is one of NOTCH signaling pathway receptors, which is branching in various cell functions including proliferation, differentiation and apoptosis. Various combinations

of **NOTCH** signaling pathway receptors and ligands might lead to different cellular responses (Andersson et al. 2011). In the present study, the expression of NOTCH1 was higher in those embryos exposed to oxidative stress coupled with elevated NRF2 transcript, as previously has been shown (Zhao et al. 2016). The upregulation of NOTCH1 under high oxygen level is attributed to cell apoptosis prevention via suppression of apoptosis signal-regulating kinase 1 (ASK1), and then preventing the p38 MAPK signaling pathway (Mo et al. 2013). This was more pronounced in embryos produced *in vitro* (Heras et al. 2016). Consistent with this finding, in the current study higher expression of NOTCH1 was found in female embryos exposed to oxidative stress than male counterparts (Figure 3.4). This phenomenon is believed to be deemed a reaction to the high level of apoptosis induced by the expression of E2F1. E2F1 is one isoform of E2Fs family involved in the regulation of cell cycle and apoptosis (Crosby and Almasan 2004). Consequently, the reduction in total cell number (Figure 3.3) in female embryos derived from high oxygen level was coupled with upregulation of E2F1 compared to male ones. On the contrary, Riverso and his colleagues reported the positive impact of E2F1 in promoting cancer cell proliferation via direct binding and regulation of KLF4 (Riverso et al. 2017).

KLF4 belongs to the KLFs transcription factor family that is involved in the regulation of proliferation, apoptosis and differentiation of various cell types (Miao et al. 2017) as well as stem cell fate in coordination with NANOG and POU5F1 (Chan et al. 2009). In the current study, we found that the higher expression of KLF4 was accompanied by differential expression of KLF4 target genes under high oxygen tension namely; POU5F1 and GATA4 (Figure 3.7). This could explain a disturbing embryonic stemness pattern at higher oxygen level (Jang et al. 2014), which was remarkable in male embryos. Irrespective of the sex of bovine embryo, transcript analysis of blastocysts showed upregulation of pluripotency-related genes (NANOG and SOX2), but not POU5F1 in response to oxidative stress (Leite et al. 2017). However, the mRNA level of GATA4 was upregulated in male embryos cultured either in 5% or 20% oxygen level compared to female counterparts.

The EVs are supposed to be involved in serval biological functions including embryo-maternal communication (Saadeldin et al. 2015; Szekeres-Bartho et al. 2018). Their size as well as concentration could vary according to cellular physiological status. Accordingly, our results indicated that the higher number and bigger size EV (Figure

3.8C) were found in the spent media of male embryos cultured under high oxygen level. Similar results were obtained in our previous study in granulosa cells exposed to oxidative stress (Saeed-Zidane et al. 2017). In contrast, the female embryos exhibited lower number and bigger size EVs upon exposure to oxidative stress. A possible explanation for this sexual dimorphic phenotype can be differences in the expression of genes involved in biogenesis and release of EVs (Hessvik and Llorente 2018; Kowal et al. 2014). Accordingly, the mRNA expression pattern of those genes revealed that ALIX and VPS4B were significantly increased in male embryos exposed to oxidative stress condition (Figure 3.9). However, female embryos showed higher expression of ALIX transcript only. Additionally, the mRNA expression level of RAB11FIP1 and RAB27A, responsible for EVs secretion (Kowal et al. 2014), showed an upregulation in male embryos exposed to oxidative stress compared to 5% oxygen level and/or female counterparts. This may indicate the sexual dimorphic physiological need in biogenesis and secretion of EVs.

It is not only the number of EVs released into extracellular space but also the molecular cargo of those EVs, which was different between male and female embryos exposed to oxidative stress. As shown in Figure 3.10, EVs from male embryos cultured at higher oxygen level contained higher level of NRF2 and SOD1 transcripts compared to those cultured under low oxygen level, which might be beneficial for the development of embryos (Pavani et al. 2018). The role of EVs not only in cell-cell communication (Saeed-Zidane et al. 2017) but also in removal of undesirable cellular molecules (Takahashi et al. 2017). Contrary to cellular expression, the NOTCH1 was increased in EVs derived from male embryos exposed to oxidative stress compared to female counterparts, which may indicate the selectivity of cargo molecules to be exported by EVs (Bhome et al. 2018; Hinger et al. 2018) to facilitate cell to cell communication or maintain of cellular homeostasis. Herein, we can summarize that exposing preimplantation bovine embryos to oxidative stress compromised the transformation of blastocysts irrespective of embryo sex, due to increasing intracellular ROS accumulation, which in turn leads to increase apoptosis and delay of differentiation in response to dysregulating of the TFs related to stress response, development, differentiation and apoptosis. Interestingly, the reduction in blastocyst formation and total cell count was more pronounced in female embryos exposed to oxidative stress compared to male counterparts, which was accompanied with alteration in cellular expression and subsequent release of TFs through EVs. Taken

together, exposure either male or female embryos to oxidative stress altered their TFs expression pattern coupled with reduction in development and delaying in differentiation. However, the male embryos are more tolerant to oxidative stress than female counterparts via activation of NRF2 and their downstream antioxidant genes at cellular and extracellular levels, which partially regulate other TFs involved in apoptosis, differentiation and development leading to skewed sex ratio toward male embryos.

3.6 Acknowledgment

Conflicts of interest

The authors declare that they have no conflicts of interest.

3.7 References

Amin A, Gad A, Salilew-Wondim D, Prastowo S, Held E, Hoelker M, Rings F, Tholen E, Neuhoff C, Looft C, Schellander K, Tesfaye D (2014) Bovine embryo survival under oxidative-stress conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway. Mol Reprod Dev 81:497-513

Anderson HC, Mulhall D, Garimella R (2010) Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. Lab Invest 90:1549-1557

Andersson ER, Sandberg R, Lendahl U (2011) Notch signaling: simplicity in design, versatility in function. Development 138:3593-3612

Andrisse S, Koehler RM, Chen JE, Patel GD, Vallurupalli VR, Ratliff BA, Warren DE, Fisher JS (2014) Role of GLUT1 in regulation of reactive oxygen species. Redox Biol 2:764-771

Beninson LA, Brown PN, Loughridge AB, Saludes JP, Maslanik T, Hills AK, Woodworth T, Craig W, Yin H, Fleshner M (2014) Acute stressor exposure modifies plasma exosome-associated heat shock protein 72 (Hsp72) and microRNA (miR-142-5p and miR-203). PLoS One 9:e108748

Bermejo-Alvarez P, Lonergan P, Rizos D, Gutierrez-Adan A (2010a) Low oxygen tension during IVM improves bovine oocyte competence and enhances anaerobic glycolysis. Reprod Biomed Online 20:341-349

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2008) Epigenetic differences between male and female bovine blastocysts produced in vitro. Physiol Genomics 32:264-272

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2010b) Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proc Natl Acad Sci U S A 107:3394-3399

Bewicke-Copley F, Mulcahy LA, Jacobs LA, Samuel P, Akbar N, Pink RC, Carter DRF (2017) Extracellular vesicles released following heat stress induce bystander effect in unstressed populations. J Extracell Vesicles 6:1340746

Bhome R, Del Vecchio F, Lee GH, Bullock MD, Primrose JN, Sayan AE, Mirnezami AH (2018) Exosomal microRNAs (exomiRs): Small molecules with a big role in cancer. Cancer Lett 420:228-235

Bryan HK, Olayanju A, Goldring CE, Park BK (2013) The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. Biochem Pharmacol 85:705-717

Cagnone G, Sirard MA (2016) The embryonic stress response to in vitro culture: insight from genomic analysis. Reproduction 152:R247-R261

Campoy I, Lanau L, Altadill T, Sequeiros T, Cabrera S, Cubo-Abert M, Perez-Benavente A, Garcia A, Borros S, Santamaria A, Ponce J, Matias-Guiu X, Reventos J, Gil-Moreno A, Rigau M, Colas E (2016) Exosome-like vesicles in uterine aspirates: a comparison of ultracentrifugation-based isolation protocols. J Transl Med 14:180

Chan KK, Zhang J, Chia NY, Chan YS, Sim HS, Tan KS, Oh SK, Ng HH, Choo AB (2009) KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells. Stem Cells 27:2114-2125

Chiang CY, Chen C (2019) Toward characterizing extracellular vesicles at a single-particle level. J Biomed Sci 26:9

Colgan SM, Tang D, Werstuck GH, Austin RC (2007) Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2. Int J Biochem Cell Biol 39:1843-1851

Crosby ME, Almasan A (2004) Opposing roles of E2Fs in cell proliferation and death. Cancer Biol Ther 3:1208-1211

Daemen S, Kutmon M, Evelo CT (2013) A pathway approach to investigate the function and regulation of SREBPs. Genes Nutr 8:289-300

Dallemagne M, Ghys E, De Schrevel C, Mwema A, De Troy D, Rasse C, Donnay I (2018) Oxidative stress differentially impacts male and female bovine embryos depending on the culture medium and the stress condition. Theriogenology 117:49-56

de la Torre Gomez C, Goreham RV, Bech Serra JJ, Nann T, Kussmann M (2018) "Exosomics"-a review of biophysics, biology and biochemistry of exosomes with a focus on human breast milk. Front Genet 9:92

Farin PW, Farin CE (1995) Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. Biol Reprod 52:676-682

Fischer B, Bavister BD (1993) Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil 99:673-679

Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D (2012) Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. Biol Reprod 87:100

Ghys E, Dallemagne M, De Troy D, Sauvegarde C, Errachid A, Donnay I (2016) Female bovine blastocysts are more prone to apoptosis than male ones. Theriogenology 85:591-600

Gómez E, Carrocera S, Martin D, Herrero P, Canela N, Muñoz M (2018) Differential release of cell-signaling metabolites by male and female bovine embryos cultured in vitro. Theriogenology 114:180-184

Gutiérrez-Adán A, Granados J, Pintado B, De La Fuente J (2001) Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. Reprod Fertil Dev 13:361-365

Heras S, De Coninck DI, Van Poucke M, Goossens K, Bogado Pascottini O, Van Nieuwerburgh F, Deforce D, De Sutter P, Leroy JL, Gutierrez-Adan A, Peelman L, Van Soom A (2016) Suboptimal culture conditions induce more deviations in gene expression in male than female bovine blastocysts. BMC Genomics 17:72

Hessvik NP, Llorente A (2018) Current knowledge on exosome biogenesis and release. Cell Mol Life Sci 75:193-208

Hinger SA, Cha DJ, Franklin JL, Higginbotham JN, Dou Y, Ping J, Shu L, Prasad N, Levy S, Zhang B, Liu Q, Weaver AM, Coffey RJ, Patton JG (2018) Diverse long RNAs are differentially sorted into extracellular vesicles secreted by colorectal cancer cells. Cell Rep 25:715-725 e714

Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H (1999) High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 52:683-700

Hung WT, Hong X, Christenson LK, McGinnis LK (2015) Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biol Reprod 93:117

Hung WT, Navakanitworakul R, Khan T, Zhang P, Davis JS, McGinnis LK, Christenson LK (2017) Stage-specific follicular extracellular vesicle uptake and regulation of bovine granulosa cell proliferation. Biol Reprod 97:644-655

Hylan D, Giraldo AM, Carter JA, Gentry GT, Jr., Bondioli KR, Godke RA (2009) Sex ratio of bovine embryos and calves originating from the left and right ovaries. Biol Reprod 81:933-938

Iwata H (2012) Factors affecting the primary sex ratio in cows. J Mamm Ova Res 29:96–102

Iwata H, Kimura K, Hashimoto S, Ohta M, Tominaga K, Minami N (2002) Role of G6PD activity on sex ratio and developmental competence of bovine embryos under oxidative stress. J Reprod Develop 48:447–453

Jang J, Wang Y, Kim HS, Lalli MA, Kosik KS (2014) Nrf2, a regulator of the proteasome, controls self-renewal and pluripotency in human embryonic stem cells. Stem Cells 32:2616-2625

Jolly RD, Thompson KG, Winchester BG (1975) Bovine mannosidosis--a model lysosomal storage disease. Birth Defects Orig Artic Ser 11:273-278

Kadenbach B, Arnold S, Lee I, Huttemann M (2004) The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. Biochim Biophys Acta 1655:400-408

Kamisako T, Tanaka Y, Kishino Y, Ikeda T, Yamamoto K, Masuda S, Ogawa H (2014) Role of Nrf2 in the alteration of cholesterol and bile acid metabolism-related gene expression by dietary cholesterol in high fat-fed mice. J Clin Biochem Nutr 54:90-94

Kelley RL, Gardner DK (2016) Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. Reprod Biomed Online 33:537-549

Kowal J, Tkach M, Thery C (2014) Biogenesis and secretion of exosomes. Curr Opin Cell Biol 29:116-125

Lawson C, Vicencio JM, Yellon DM, Davidson SM (2016) Microvesicles and exosomes: new players in metabolic and cardiovascular disease. J Endocrinol 228:R57-71

Leite RF, Annes K, Ispada J, de Lima CB, Dos Santos EC, Fontes PK, Nogueira MFG, Milazzotto MP (2017) Oxidative stress alters the profile of transcription factors related to early development on in vitro produced embryos. Oxid Med Cell Longev 2017:1502489

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408

Martindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. Cancer Biol Ther 192:1-15

Miao X, Wu X, Shi W (2017) MicroRNA-346 regulates neural stem cell proliferation and differentiation by targeting KLF4. Am J Transl Res 9:5400-5410

Mo JS, Yoon JH, Ann EJ, Ahn JS, Baek HJ, Lee HJ, Kim SH, Kim YD, Kim MY, Park HS (2013) Notch1 modulates oxidative stress induced cell death through suppression of apoptosis signal-regulating kinase 1. Proc Natl Acad Sci U S A 110:6865-6870

Navakanitworakul R, Hung WT, Gunewardena S, Davis JS, Chotigeat W, Christenson LK (2016) Characterization and small RNA content of extracellular vesicles in follicular fluid of developing bovine antral follicles. Sci Rep 6:25486

Oliveira CS, Saraiva NZ, de Lima MR, Oliveira LZ, Serapiao RV, Garcia JM, Borges CA, Camargo LS (2016) Cell death is involved in sexual dimorphism during preimplantation development. Mech Dev 139:42-50

Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL (1986) Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 25:591-600

Pavani KC, Hendrix A, Van Den Broeck W, Couck L, Szymanska K, Lin X, De Koster J, Van Soom A, Leemans B (2018) Isolation and characterization of functionally active extracellular vesicles from culture medium conditioned by bovine embryos in vitro. Int J Mol Sci 20:

Pérez-Cerezales M, Ramírez MA, Fernández-González R, Rizos D, Lonergan P, Pintado B, Gutiérrez-Adán A (2005) Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. Mol Reprod Dev 72:502-510

Pérez-Cerezales S, Ramos-Ibeas P, Rizos D, Lonergan P, Bermejo-Alvarez P, Gutiérrez-Adán A (2018) Early sex-dependent differences in response to environmental stress. Reproduction 155:R39-R51

Rattanasuk S, Parnpai R, Ketudat-Cairns M (2011) Multiplex polymerase chain reaction used for bovine embryo sex determination. J Reprod Dev 57:539-542

Riverso M, Montagnani V, Stecca B (2017) KLF4 is regulated by RAS/RAF/MEK/ERK signaling through E2F1 and promotes melanoma cell growth. Oncogene 36:3322-3333

Rosenfeld CS, Roberts RM (2004) Maternal diet and other factors affecting offspring sex ratio: a review. Biol Reprod 71:1063-1070

Saadeldin IM, Oh HJ, Lee BC (2015) Embryonic-maternal cross-talk via exosomes: potential implications. Stem Cells Cloning 8:103-107

Saeed-Zidane M, Linden L, Salilew-Wondim D, Held E, Neuhoff C, Tholen E, Hoelker M, Schellander K, Tesfaye D (2017) Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress. PLoS One 12:e0187569

Schellander K, Fuhrer F, Brackett BG, Korb H, Schleger W (1990) In vitro fertilization and cleavage of bovine oocytes matured in medium supplemented with estrous cow serum. Theriogenology 33:477-485

Shimano H (2001) Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. Prog Lipid Res 40:439-452

Siqueira LG, Hansen PJ (2016) Sex differences in response of the bovine embryo to colony-stimulating factor 2. Reproduction 152:645-654

Ståhl AL, Johansson K, Mossberg M, Kahn R, Karpman D (2019) Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. Pediatr Nephrol 34:11-30

Stanton JA, Macgregor AB, Green DP (2003) Gene expression in the mouse preimplantation embryo. Reproduction 125:457-468

Sun D, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, Barnes S, Grizzle W, Miller D, Zhang HG (2010) A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. Mol Ther 18:1606-1614

Szekeres-Bartho J, Šućurović S, Mulac-Jeričević B (2018) The role of extracellular vesicles and PIBF in embryo-maternal immune-interactions. Front Immunol 9:2890

Takahashi A, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S, Takasugi M, Watanabe S, Kanemaki MT, Obuse C, Hara E (2017) Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. Nat Commun 8:15287

Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR, King WA (1991) Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. J Reprod Fertil 93:125-132

Van Soom A, Yuan YQ, Peelman LJ, de Matos DG, Dewulf J, Laevens H, de Kruif A (2002) Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. Theriogenology 57:1453-1465

Wale PL, Gardner DK (2016) The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. Hum Reprod Update 22:2-22

Xu KP, Yadav BR, King WA, Betteridge KJ (1992) Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. Mol Reprod Dev 31:249-252

Yoon SB, Choi SA, Sim BW, Kim JS, Mun SE, Jeong PS, Yang HJ, Lee Y, Park YH, Song BS, Kim YH, Jeong KJ, Huh JW, Lee SR, Kim SU, Chang KT (2014) Developmental competence of bovine early embryos depends on the coupled response between oxidative and endoplasmic reticulum stress. Biol Reprod 90:104

Zhang DD (2006) Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev 38:769-789

Zhao Q, Mao A, Yan J, Sun C, Di C, Zhou X, Li H, Guo R, Zhang H (2016) Downregulation of Nrf2 promotes radiation-induced apoptosis through Nrf2 mediated Notch signaling in non-small cell lung cancer cells. Int J Oncol 48:765-773

Table 3.1. The list of primers sequence used for quantitative real-time PCR

Gene	Accession number	Primer sequences[*]	Product size (bp)
ACTB	NM_173979	F: 5´-TGTCCACCTTCCAGCAGAT-3´	249
		R: 5´-TCACCTTCACCGTTCCAGT-3´	
GAPDH	NM_001034034	F: 5´-CCCAGAATATCATCCCTGCT-3´	185
		R: 5´-CTGCTTCACCACCTTCTTGA-3´	
18S	NR_036642	F: 5´-CGCAGCTAGGAATAATGGAA-3´	210
		R: 5´-TCTGATCGTCTTCGAACCTC-3´	
KLF4	NM_001105385.1	F: 5´-CAAACCAAAGAGGGGAAGAC-3´	175
		R: 5´-AAACTTCCACCCACAACCAT-3´	
NOTCH1	XM_024999642.1	F: 5´-AGCAACATTAACGAGTGCCA-3´	125
		R: 5´-TCCAGGTTGATCTCGCAGTT-3´	
E2F1	NM_001206079.1	F: 5´-TCTTGGAATTACTGAGCCGC-3´	209
		R: 5´-CTGGGTCAATCCTTCAAGCC-3´	
SOD1	NM_174615	F: 5´-AGAGGCATGTTGGAGACCTG -3´	189
		R: 5´-CAGCGTTGCCAGTCTTTGTA -3´	
CAT	NM_001035386.1	F: 5´-TGGGACCCAACTATCTCCAG-3´	178
		R: 5´-AAGTGGGTCCTGTGTTCCAG-3´	
SREBF2	NM_001205600.2	F: 5´-TTCCTCTGCCTCTCCTTTAAC-3´	183
		R: 5´-CACGATCATGCCATTCACCA-3´	
NRF2	NM_001011678	F: 5´-CCCAGTCTTCACTGCTCCTC-3´	165
		R: 5´-TCAGCCAGCTTGTCATTTTG-3´	
GATA4	NM_001192877.1	F: 5´-CTGCTCTGTCTGGGAATGAC-3´	228
		R: 5´-GACTTCTCGTCTCTTGTCGG-3´	
ALIX	XM_005222590.4	F: 5´-GAGTACCACCAGTCTATCCT-3´	225
		R: 5´-AGGGTCCAGATCTTTAAGGT-3´	
VPS4B	NM_001076156.1	F: 5´-TTATAGAGCGACCCAATGTG-3´	208
		R: 5´-GAGTTATTTGCTTCTGTGGC-3´	
TSAP6	NM_001304338.1	F: 5´-CAAGGGATGGAAATAGGCAG-3´	222
		R: 5´-CAAAGTTGTAGGCGTAGAAG-3´	
RAB11FIP1	XM_005226137.4	F: 5´-TCGTGAGTGAGAACTTGATC-3´	176
		R: 5´-GTTGTCGATGTAGTCTTCCA-3´	
RAB35	NM_001098127.1	F: 5´-ATCACCTCCACGTATTATCG-3´	233
		R: 5´-CTGGTCTCAAACAACTGGAT-3´	
RAB27A	NM_001101270.1	F: 5´-CTATGGGGTTTCTTCTGCTT-3´	196
		R: 5´-ACTCCGTATTTCTCTGCAAG-3´	
POU5F1	NM_174580.3	F: 5´-GATATACCCAGGCCGATGTG-3´	240
		R: 5´-CTCGGTTCTCGATACTCGTC-3´	

[*]F: Forward, R: Reverse
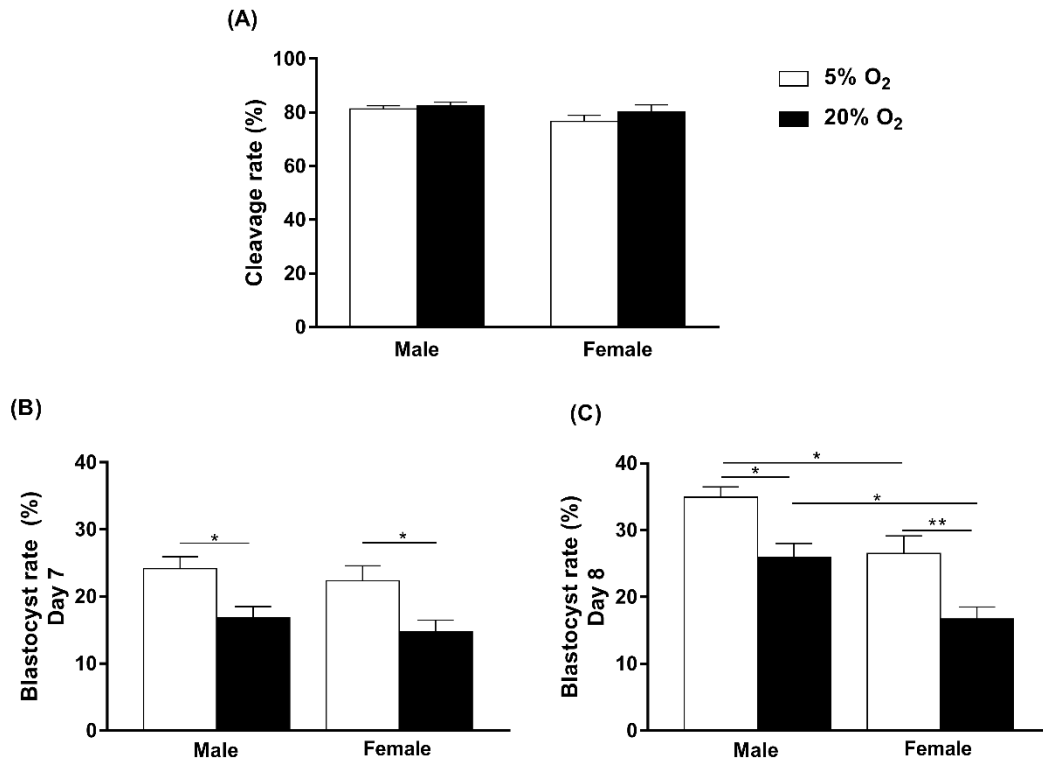
Figure 3.1. The cleavage (A) and blastocyst rates at day 7 (B) and day 8 (C) of male and female preimplantation embryos cultured under 5% (white bar) or 20% (black bar) oxygen levels. Data are represented as mean ± SEM of four independent biological replicates
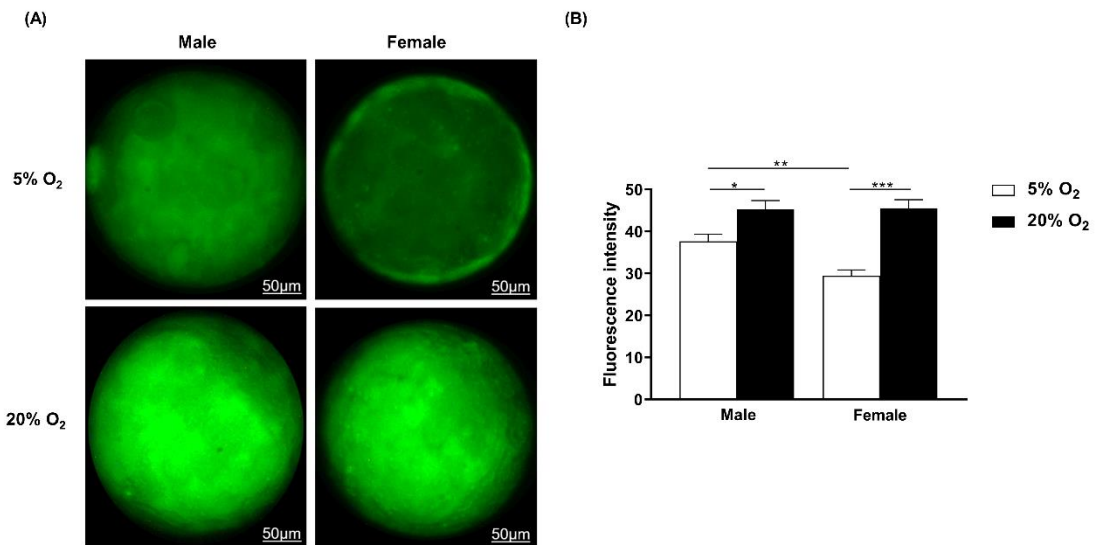
Figure 3.2. Intracellular ROS accumulation (A) and fluorescence intensity of ROS signal (B) of male and female preimplantation embryos derived from 5% (white bar) and 20% (black bar) oxygen levels. 50 µm indicates the scale bar, values of fluorescence intensity are represented as means ± SEM (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$*). ROS, reactive oxygen species
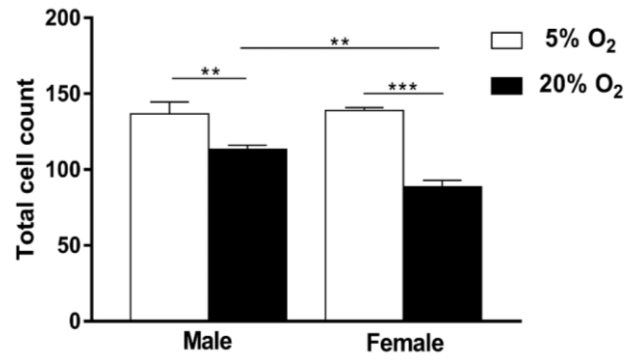
Figure 3.3. Total cell number of male and female blastocysts derived from 5% (white bar) and 20% (black bar) oxygen levels. Data are represented as means ± SEM *(**p≤0.01, ***p≤0.001)*
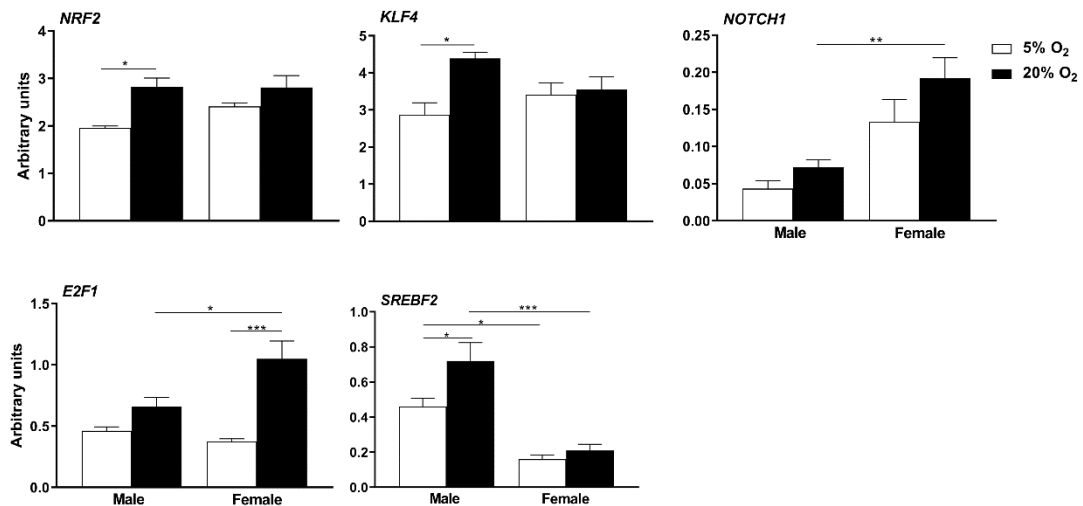
Figure 3.4. The mRNA expression pattern of transcription factors (NRF2, KLF4, NOTCH1, E2F1 and SREBF2) in male and female blastocysts cultured in 5% or 20% oxygen level. Expression was compared between 5% (white bar) and 20% oxygen levels within and between sexes. Data are represented as means ± SEM (*p≤0.05, **p≤0.01, ***p≤0.001*). The arbitrary units were multiplied by 100. E2F1, E2F transcription factor 1; KLF4, Krüppel-like factor 4; NFE2L2, nuclear factor (erythroid-derived 2) factor 2; NOTCH1, notch receptor 1; SREBF2, sterol regulatory element-binding transcription factor 2
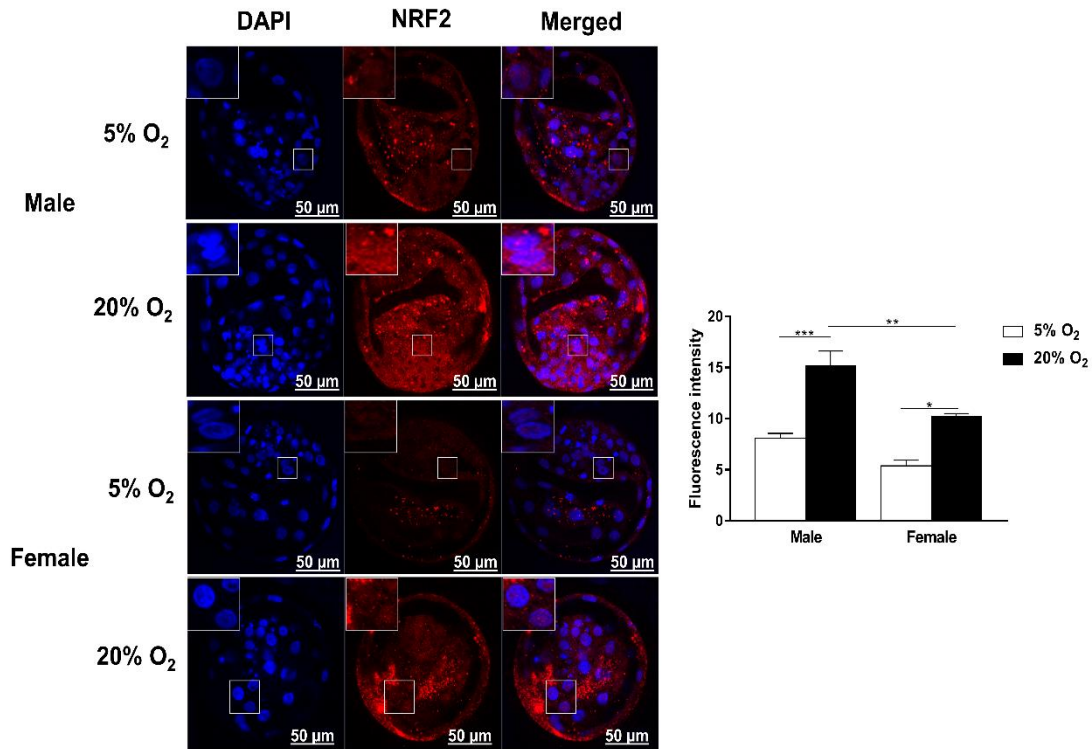
Figure 3.5. Detection and immunolocalization of NRF2 protein in male and female blastocyst-stage embryos derived from 5% or 20% oxygen levels. Red color reveals the localization of the NRF2 protein, while the blue color is nuclear staining (DAPI). 50 µm indicates the scale bar. The bars represent means ± SEM *(*p≤0.05, **p≤0.01, ***p≤0.001)* of fluorescence signals as quantified by ImageJ software. NRF2, nuclear factor (erythroid-derived 2) factor 2
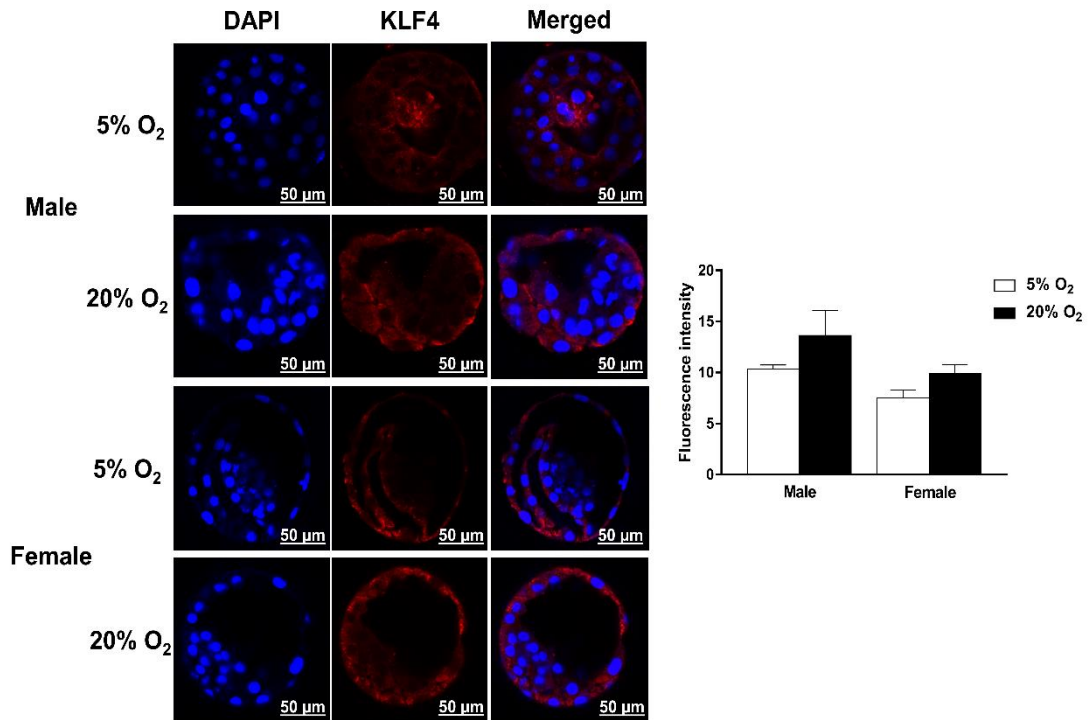
Figure 3.6. Detection and immunolocalization of KLF4 protein in male and female blastocyst-stage embryos derived from 5% or 20% oxygen levels. Red color reveals the localization of the KLF4 protein, while the blue color is nuclear staining (DAPI). 50 µm indicates the scale bar. The bars represent means ± SEM of fluorescence intensity of the protein as quantified by ImageJ software. KLF4, Krüppel-like factor 4
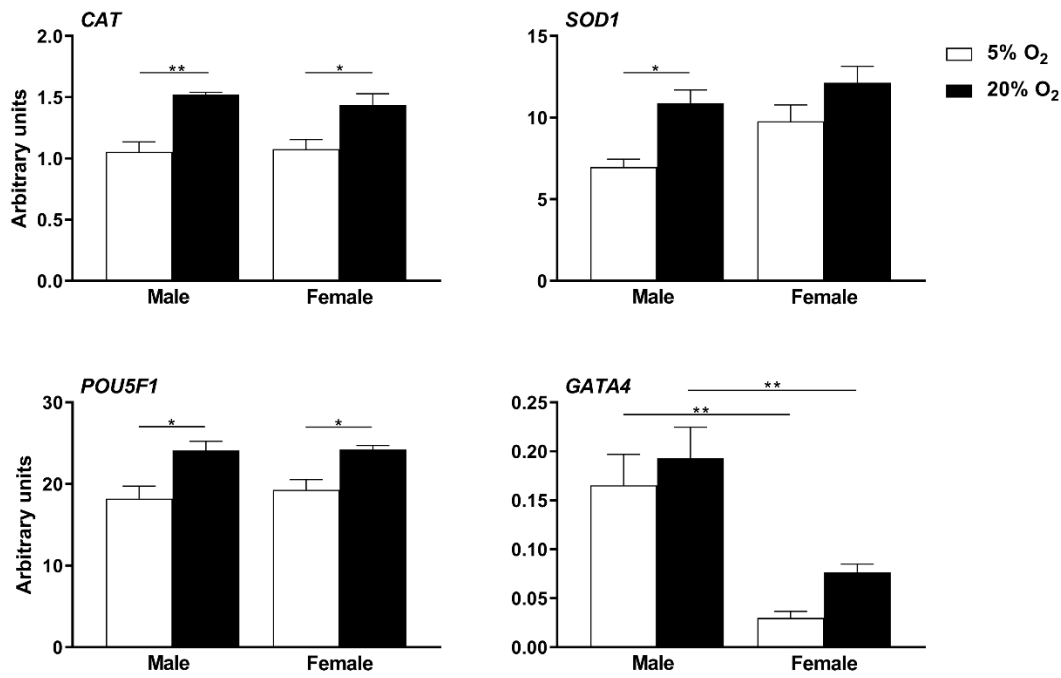
Figure 3.7. The mRNA expression pattern of genes related to antioxidant system (CAT and SOD1) and differentiation (POU5F1 and GATA4) in male and female blastocysts cultured under 5% or 20% oxygen level. Expression was compared between 5% (white bar) and 20% oxygen levels under the same sex and between sexes. Data are represented as means ± SEM (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$*). The arbitrary units were multiplied by 100. CAT, catalase; GATA4, GATA binding protein 4; POU5F1, POU class 5 homeobox 1; SOD1, superoxide dismutase 1
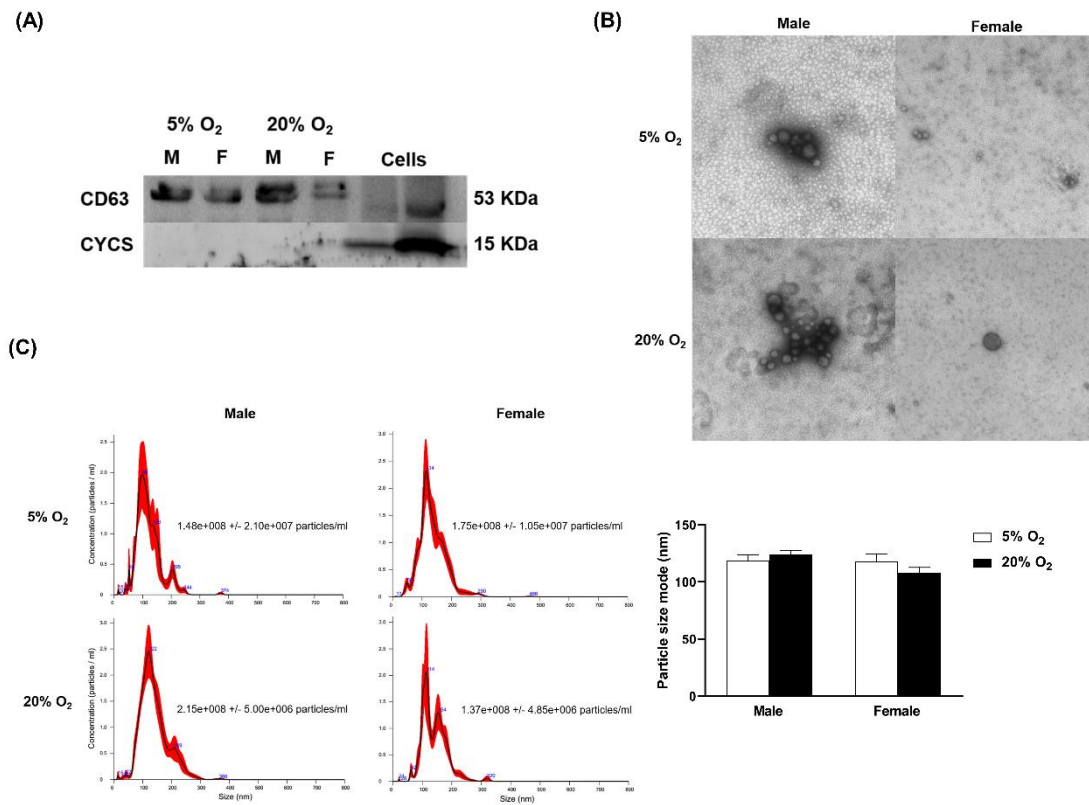
Figure 3.8. Characterization of extracellular vesicles (EVs) derived from *in vitro* spent media of male and female embryos cultured under 5% or 20% oxygen levels. The purity and identity of isolated EVs were evaluated using Western blot (A), electron microscopy (B), and nanoparticles tracking analysis (C). The letters M and F indicate male and female, respectively. The white bars indicate 5% oxygen level and the black bars indicate 20% oxygen level
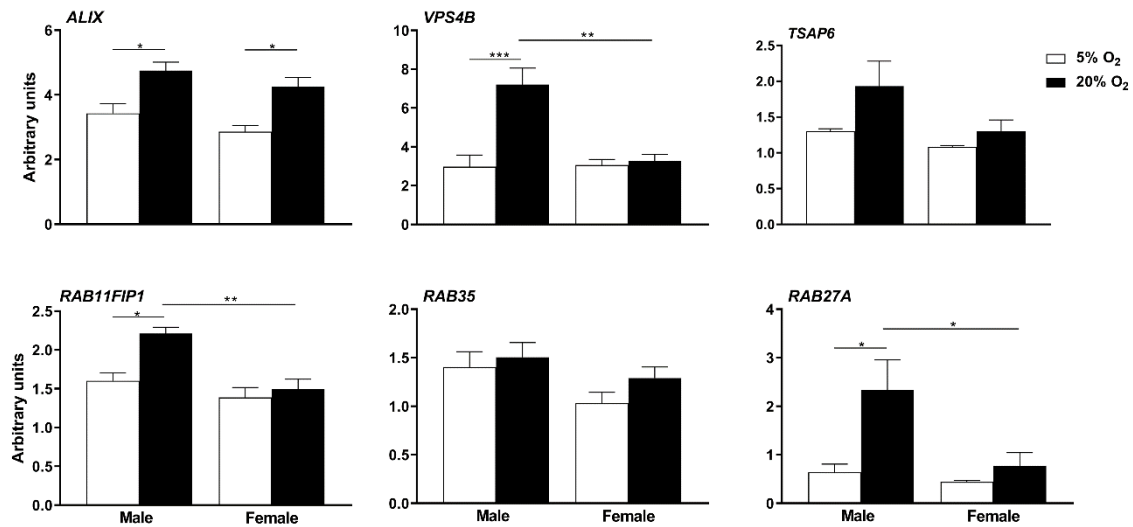
Figure 3.9. The mRNA expression pattern of extracellular vesicles biogenesis (ALIX, VPS4B, and TSAP6) and secretion (RAB11FIP1, RAB35, and RAB27A) genes in male and female blastocysts cultured under 5% or 20% oxygen levels. Expression was compared between 5% (white bar) and 20% oxygen levels under the same sex and between sexes. Data are represented as means ± SEM (*$p \leq 0.05$*, **$p \leq 0.01$*, ***$p \leq 0.001$*). The arbitrary units were multiplied by 100. RAB11FIP1, RAB11 family interacting protein 2; TSAP6, STEAP3 metalloreductase; VPS4B, vacuolar protein sorting 4 homolog B
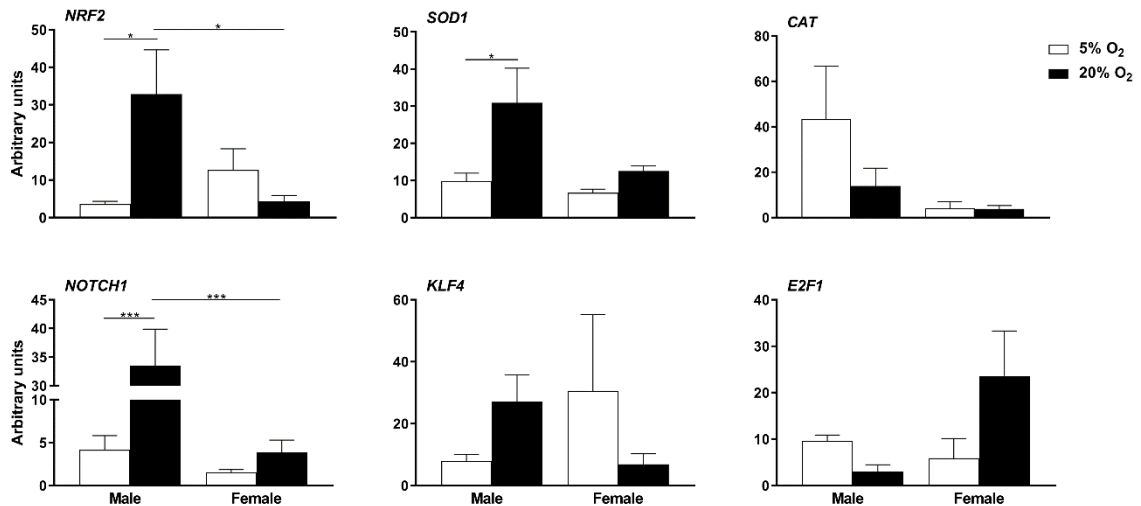
Figure 3.10. The mRNA expression pattern of extracellular vesicles-coupled transcription factors (NRF2, KLF4, NOTCH1 and E2F1) and antioxidant genes (CAT and SOD1) released into culture media of male and female embryos cultured under 5% or 20% oxygen levels. Expression was compared between 5% (white bar) and 20% (black bar) oxygen levels under the same sex and between sexes. The mRNA level was measured by qRT-PCR and normalized by the geometric means of three housekeeping genes (ACTB, GAPDH and 18S). Data are represented as means ± SEM (*$p \leq 0.05$, ***$p \leq 0.001$). The arbitrary units were multiplied by 100. CAT, catalase; E2F1, E2F transcription factor 1; KLF4, Krüppel-like factor 4; NFE2L2, nuclear factor (erythroid-derived 2) factor 2; NOTCH1, notch receptor 1; SOD1, superoxide dismutase 1

3.8 Supplementary data
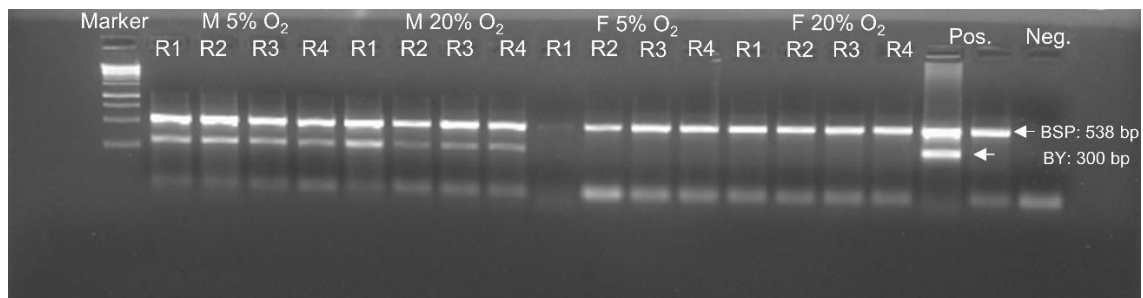
Supplementary Figure 3.1. Validation of the sex of embryos produced by sex-sorted semen using multiplex PCR primers (BSP and BY). M: males, F: female, R: number of replicates, Pos.: positive controls and Neg.: negative controls

Supplementary Table 3.1. Concentration of RNA isolated from male and female embryos generated under 5% or 20 oxygen level culture condition

Sex	Oxygen level	RNA concentration (ng/μL)
Male	5%	13.97
		7.49
		9.65
		8.46
	20%	10.02
		6.39
		8.66
		11.00
Female	5%	7.24
		7.85
		9.38
		7.24
	20%	8.88
		7.66
		7.57
		6.62

Supplementary Table 3.2. Concentration of RNA isolated extracellular vesicles of male and female embryos cultured under 5% or 20 oxygen level

Sex	Oxygen level	RNA concentration (ng/µL)
Male	5%	12.20
		11.80
		12.09
		11.52
	20%	13.81
		12.76
		12.57
		11.06
Female	5%	13.84
		11.71
		11.86
		10.82
	20%	12.36
		12.65
		11.75
		12.34

Chapter 4: General discussion

4.1 Discussion

Cattle fertility is one of the important factors in animal production and breeding strategies. However, fertility is a complex multifactorial trait, which is mainly governed by the interaction between genetics and the environment (Craig et al. 2018). In order to improve cow fertility, we have to understand the genetic background of different biological functions involved in fertility and their interaction with micro and macro environments. The most critical mammalian organ in female reproduction is the ovary, which contains follicles as a functional unit. The ovarian follicle contains the oocyte surrounded by multiple layers of somatic cells; namely granulosa and theca cells. Those cells are essential for supplying the oocyte with essential nutrients and production of steroid hormones (Dzafic et al. 2013). Only a few of growing follicles reach the ovulation stage, while the remaining undergo atresia (Shen et al. 2012). In the ovary, apoptosis is involved in several physiological aspects including follicular atresia, ovulation, luteolysis (Hussein 2005; Regan et al. 2018; Tilly 1996). The ovarian apoptosis is regulated by several hormones such as insulin-like growth factor-I (IGF-I), estrogens (E2), and gonadotropin-releasing hormone (GnRH). However, the action of GnRH in the ovary via regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) depends on the stage of the follicle, which extends from inhibition the growth of small follicle via inhibition of steroidogenesis process (Hsueh and Jones 1981) to induction of follicular atresia (Parborell et al. 2005). Three consecutive stages are believed to be involved in apoptosis process include: (1) extracellular (Fas/FasL, caspase 8) or intracellular (Bcl-2, caspase 9) signals that stimulate cell death; (2) activation of intracellular caspase 3 and 7 to enforce cell death; and (3) ultimately engulfment by neighbor cells or packed into apoptotic bodies to eliminate dead cells (Vaux and Strasser 1996).

ROS were found to play a pivotal role during atresia (Hennet et al. 2013) and ovulation processes (Shkolnik et al. 2011; Tanabe et al. 2015). The excess production of intracellular ROS above the cellular antioxidant capability is called oxidative stress (Agarwal et al. 2006). Oxidative stress could also be generated from external stressors such as inflammation (Shi et al. 2016), heat stress (Cong et al. 2017; Montilla et al. 2014) or metabolic stress (Putman et al. 2018). These stressors are believed to adversely affect the reproductive target cells (Vanselow et al. 2016) by compromising cell membrane, mitochondria activity and integrity and causes DNA damages (Kadenbach et al. 2004; Zhang et al. 2016). This is in agreement with the current study, which showed higher ROS

accumulation (Figure 2.2a), lower mitochondrial activity (Figure 2.2b) and higher DNA fragmentation (Figure 2.3) of granulosa cells in response to oxidative stress. In addition, accumulated evidence showed that high ROS levels-induced oxidative stress could disrupt the protein folding process and subsequently cause endoplasmic reticulum stress (Alemu et al. 2018; Kim et al. 2016; Malhotra et al. 2008). Similarly, exposure of granulosa cells in the current study to oxidative stress led to the upregulation of endoplasmic reticulum stress marker genes (Grp78 and Grp94) at mRNA and protein levels (Figure 2.4a and b). Consequently, this disturbance in different cellular compartments due to exposure to oxidative stress could lead to cell cycle arrest (Saeed-Zidane et al. 2017) and subsequently abnormal apoptosis (Sohel et al. 2019) in granulosa cells. Moreover, the deficiency in granulosa cell functions could reduce oocyte quality and ultimately failure of pregnancy (Lai et al. 2018). However, the endoplasmic reticulum stress has been shown to be involved in follicular atresia (Lin et al. 2012) as well as other ovarian function, such as growth and maturation of ovarian follicle as well as corpus luteum biogenesis (Huang et al. 2017), which indicates its dual role during atresia and ovulation as well.

Transcription factors (TFs) are believed to be involved in cellular adaptive response (da Silveira et al. 2017; Hellweg et al. 2016; Saeed-Zidane et al. 2017). Among them is the nuclear erythroid-derived 2-related factor 2 (NEF2L2 or NRF2), which is deemed to be the maestro of cellular adaptive response during oxidative stress, through triggering the cellular antioxidants response mechanisms. Upon higher intracellular ROS accumulation, the NRF2 dissociates from its cytosolic inhibitor Kelch-like ECH-associated protein 1 (KEAP1) then translocates to the nucleus and binds to the promoter of antioxidant genes named antioxidant response elements (ARE) (Taguchi et al. 2011). This action was found to be essential for the removal of excessive levels of ROS in various cell types including granulosa cells (Alemu et al. 2018; Saeed-Zidane et al. 2017; Sohel et al. 2019), which is in consistence with current results (Figure 2.5a and b). The high levels of NRF2 were found to be localized in mice granulosa cells as well as oocyte of secondary and antral follicles (Sindan et al. 2018). Consistently, H_2O_2-induced high intracellular ROS levels have been reported to be involved in cumulus cells expansion and ovulation process, which was inhibited by injecting a ROS scavenger into ovarian bursa namely butylated hydroxyanisole (Shkolnik et al. 2011). Accordingly, this fact indicates the potential role of high intracellular ROS accumulation-induced NRF2 expression in the ovulation

process, which is characterized as an inflammatory process in response to LH surge (Duffy et al. 2019).

The over accumulation of ROS could lead to higher expression of NRF2, which in turn results in cell apoptosis via binding to and activation of Krüppel-like family 9 (KLF9) (Zucker et al. 2014). Regarding this fact, the present study found upregulation of KLF9 (Figure 2.5c) in H_2O_2-challenged granulosa cells accompanied by lower cellular proliferation (Figure 2.9c). The family of this TF is involved in the regulation of several biological functions including apoptosis, proliferation and differentiation (Kaczynski et al. 2003). Moreover, KLF6 was found to be upregulated in H_2O_2-treated granulosa cells compared to untreated counterparts (Figure 2.5c). Consistently, the upregulation of KLF6 was found in small atretic follicles compared to healthy counterparts (Hatzirodos et al. 2014a), which indicates its role in induction of apoptosis during follicular atresia. Nevertheless, the upregulation of KLF6 and its variants were shown in response to alcohol-induced ROS accumulation and consequently modulate the expression of tumor necrosis factor-alpha (TNFα) (Urtasun et al. 2012). The expression of TNFα contributes to ovulation process through inducing apoptosis in granulosa cells of unruptured follicles along with reducing the number of ovulated oocytes (Yamamoto et al. 2015). Besides this, it was found that TNFα expression stimulated the expression of several genes, which are involved in ovulation process, proteolysis and tissue remolding in trout (Crespo et al. 2010).

The transit abundance of KLF4 expression was found to be higher in granulosa cells of the preovulatory follicle in order to induce apoptosis and cell cycle arrest in response to LH surge (Choi and Roh 2018). Therefore, its expression was found to be increased after 1 h from human chorionic gonadotropin (hCG) administration (Carletti and Christenson 2009), which may indicate that its response to LH hormone is time-dependent. Furthermore, the expression of KLF4 negatively regulated the expression of CYP19A1, which indicates its pivotal role in granulosa-luteal cells transition steroidogenesis (Choi et al. 2019). The current results were not matched with the previous evidence, where the KLF4 was downregulated at mRNA (Figure 2.5d) and protein levels (Figure 2.6a) in the H_2O_2-challenged group. Accordingly, the mRNA expression of CYP19A1 was upregulated in H_2O_2-treated cells (Figure 8a), which confirms the negative regulation of KLF4 to CYP19A1 as evidenced previously (Choi et al. 2019). On the other hand, the KLF4 was revealed to be post-transcriptionally regulated by several microRNAs.

Previous studies showed that miR-145 is predicted to target KLF4 and showed a higher expression in granulosa cells exposed to oxidative stress in order to protect granulosa cells from H_2O_2-induced apoptosis (Sohel et al. 2019; Xu et al. 2017), which could reveal that its induction is H_2O_2 dose and time-dependent.

Supporting to this notion, miR-145 was downregulated in granulosa cells of polycystic ovary syndrome (PCOS) patients but tended to be higher in their follicular fluids and serum (Naji et al. 2018). In endothelial cells, miR-92a was shown to target KLF4 under oxidative stress conditions in response to stimulation of sterol regulatory element-binding transcription factor 2 (SREBF2), which contributes to endothelial cells dysfunction under oxidative stress conditions (Chen et al. 2015). This evidence is compatible with the present results (Figure 2.5c), which showed an upregulation of SREBF2 upon exposure of granulosa cells to oxidative stress.

SREBFs are TFs family involved in the regulation of cellular fatty acids and cholesterol biosynthesis via binding of SREBF1, which has two isoforms SREBF1a as well as SREBF1c, and SREBF2 to sterol regulatory element (SRE), respectively (Daemen et al. 2013; Shimano 2001). The high similarity within the active domain among subtypes of SREBPs family leads to regulating their target genes with different efficiency (Shimano 2001). Accordingly, the depletion of SREBF2 in mice hepatocyte did not only reduce the cholesterol biogenesis but also led to reducing SREBF1c, which is responsible for fatty acids biogenesis (Rong et al. 2017). Furthermore, the depletion of SREBFs family altered the cellular lipid and protein biosynthesis via inhibition of the AKT pathway coupled with upregulation of genes linked to stress response and inflammation as well as endoplasmic reticulum stress (Griffiths et al. 2013). The endoplasmic reticulum stress had been proven to induce cellular cholesterol and lipid accumulation through increasing SREBF2 cleavage (Colgan et al. 2007) and expression of SREBF1c (Fang et al. 2013), respectively. This fact indicates the involvement of endoplasmic reticulum stress induced by higher ROS accumulation in lipid accumulation (Alemu et al. 2018; Sohel et al. 2018), which is in harmony with the current study (Figure 2.7c). However, this higher lipid accumulation was attenuated in *in vitro* culture of bovine granulosa cells supplemented with sulforaphane, as an antioxidant activator (Sohel et al. 2018).

On the other hand, the expression of SREBFs was increased in response to exogenous administration of hCG hormone and insulin via downregulation of SREBFs inhibitor; namely insulin-induced gene 1 (INSIG1) protein (Palaniappan and Menon 2009), which

may partially indicate their role in steroidogenesis. Furthermore, the treated granulosa cells with FSH showed upregulation of SREBFs and those genes involved in steroidogenesis such as STAR, CYP11A1, and CYP19A1 (Liu et al. 2009), which is in agreement with the results of this study (Figure 2.8a and b). The CYP11A1 is responsible for perpetrating the cholesterol to steroids biosynthesis pathway. CYP11A1 was recruited in antral follicles (LaVoie 2017), which similarly was found in granulosa cells in advance of LH surge (Garmey et al. 2000). Also, the overexpression of KLFs family including KLF4, KLF9, and KLF13 in granulosa cells had a negative effect on the expression of CYP11A1 (Natesampillai et al. 2008). Besides this, the expression of STAR was also observed to be insignificant until exposure of granulosa cells to LH surge (Garmey et al. 2000; LaVoie 2017). These facts could indicate that the expression of SREBFs along with steroidogenesis genes is indispensable during granulosa cells transdifferentiation in response to LH surge. Interestingly, the expression of SREBF1c could be regulated by NRF2 (Kamisako et al. 2014). Consequently, the lipogenic genes were upregulated in bovine embryos exposed to oxidative stress-induced high expression of NRF2 (Amin et al. 2014).

Regarding microarray data of granulosa cells after ovarian superstimulation, upregulation of NRF2 and other genes involved in oxidative stress response was coupled with upregulation of c-FOS (Dias et al. 2013), which is in parallel with the current results (Figure 2.10c). c-FOS is one of the activator protein-1 (AP-1) members including c-Jun, JunB, JunD, c-Fos, FRA1 and FRA2, which are regulated via c-Jun NH2-terminal kinase (JNK) AP-1 (Yin et al. 2009). Its expression is positively associated with FSH (Delidow et al. 1992) and showed a declined pattern 6 h post-LH surge (Gilbert et al. 2011). Furthermore, the relative abundance of c-FOS increased the apoptosis of granulosa cells upon exposure to mycotoxin (Guerrero-Netro et al. 2015) as well as in atretic follicles (Hatzirodos et al. 2014a; Hatzirodos et al. 2014b), indicating its role in activating apoptosis pathways under stress conditions (Preston et al. 1996). These facts are in the same line with the current study, which showed an increased mRNA level of c-FOS in response to oxidative stress (Figure 2.5c).

Knockout of retinoblastoma protein (RB), which is responsible for the regulation of cellular proliferation and survival, in mice resulted in the upregulation of c-FOS and E2F1 in the preantral follicle (Andreu-Vieyra et al. 2008). The pRB had been found to regulate the expression of E2F1-induced apoptosis in a different manner than other members of

E2Fs family (Dick and Dyson 2003). E2Fs are family of TFs involved in regulation of cell cycle progression as well as apoptosis under certain conditions. This family contains eight genes involved in activation or suppression of cell cycle. E2F1-3 are served as cell cycle activators, whereas E2F4-5 are involved in cell cycle suppression (Iaquinta and Lees 2007). Additionally, it is involved in regulation of fatty acids and glucose metabolism (Hsieh et al. 2008) as well as regulation of genes involved in steroidogenesis (Sharma et al. 2009). On the other hand, the downregulation of pRB was observed in response to NRF2 knockdown leading to inhibition of lung cancer cell proliferation, which indicates its pivotal role coupled with E2Fs family during cell cycle progression (Homma et al. 2009). This could be interpreted the reduction of E2F1 under NRF2-silencing in the current study (Figure 2.10c).

E2F1 was found to be enriched in granulosa cells of growing follicles in prepubertal compared to adult sheep counterparts indicating its role in cell proliferation (Wu et al. 2017). However, it was reviewed that the overexpression of E2F1 in different models either tissue culture cells or transgenic animals led to induction of apoptosis, which has been inhibited by inhibition of E2F1 (Ginsberg 2002). Due to p53 and p53 upregulated modulator of apoptosis (PUMA) contain a binding site of E2F1 on their promotor, the overexpression of E2F1 coupled with PUMA led to apoptosis via regulation of p53 in mouse treated with the herbicide Atrazine (Sharma et al. 2014). On the other hand, the p53 was found to be negatively regulated MYC, FOS, and E2F1-3 (Douville and Sirard 2014). Interestingly, the previous study in tumor cells revealed a positive correlation between E2Fs and NOTCHs in terms of expression, which was confirmed by the presence of E2F-binding sites in NOTCHs promotor (Viatour et al. 2011).

NOTCHs is a signaling pathway consisting of four receptors (NOTCH1, NOTCH2, NOTCH3, and NOTCH4) and five ligands (JAG1, JAG2, DLL1, DLL3 and DLL4), which bind together and produce various combinations of receptors and ligands leading to different cellular response (Andersson et al. 2011). This pathway is involved in several biological functions including cell proliferation, differentiation and apoptosis. Besides this, it has a pivotal role in the regulation of fetal-maternal communication during implantation (Chu et al. 2011). Once receptor activation occurred, the NOTCH intracellular domain (NICD) is formed and then translocated to the nucleus, where it binds to specific DNA sites like CLS, Mastermind (Mam) and transcriptional co-activators in order to activate or suppress their target genes (Bray 2006; Kopan and Ilagan 2009).

In the mammalian ovary, the depletion of JAG1 led to the suppression of genes involved in steroids biosynthesis, which in turn resulted in reduction of estrogen and progesterone levels. Additionally, STAR, which is responsible for cholesterol transporting to inner mitochondria membrane, was amongst downregulated genes in response to JAG1 knockdown (Prasasya and Mayo 2018), which could confirm the upregulation of STAR (Figure 2.8a) in the present study in response to NOTCH1 (Figure 2.5c) under oxidative stress conditions. This indicates the pivotal role of NOTCH signaling in promoting granulosa cells differentiation in the preovulatory follicle. Nonetheless, the dysregulation of the NOTCH signaling pathway led to reduction in primordial follicles (Trombly et al. 2009; Vanorny et al. 2014; Xu and Gridley 2013). Moreover, appearing of multioocytic follicles had been reported in response to knockout of NOTCH2 and JAG1 in mice granulosa cells and oocytes, respectively, which in turn led to reduction of fertility (Vanorny et al. 2014; Xu and Gridley 2013). On the other hand, the downregulation of NRF2 led to increasing intracellular ROS accumulation and subsequently increasing cell apoptosis in lung cancer cells, which was accompanied by the downregulation of NOTCH1 (Zhao et al. 2016). This evidence highlighted the potential regulation of NOTCH1 via NRF2 (Figure 2.10c). From this point of view, the upregulation of NOTCH1 under oxidative stress conditions is required to prevent oxidative stress-induced cell apoptosis via suppression of apoptosis signal-regulating kinase 1 (ASK1) and then preventing the p38 MAPK signaling pathway (Mo et al. 2013).

The disturbance in granulosa cells functions in response to oxidative stress could negatively impact the oocyte quality, and consequently reduce the fertilization rate, cleavage rate, and ultimately the quality of the embryo produced (Lai et al. 2018). Many stressors during embryo *in vitro* production including pH, culture media, temperature, and oxygen concentration could disturb the balance between prooxidants and antioxidants leading to oxidative stress (Cagnone and Sirard 2016; Sakatani 2017; Takahashi 2012; Wale and Gardner 2016). In this respect, previous evidence had been indicated that the oxygen level (20% O_2), which is widely used in *in vitro* embryo production, is greater than the oxygen level (1.5-8.7%) in the reproductive tract of most mammals (Fischer and Bavister 1993). Accordingly, the transcriptome analysis of bovine blastocyst showed that array of genes including TFs were altered in response to *in vitro* conditions compared to *in vivo* counterparts especially when the embryos were *in vitro* culture until embryonic

genome activation. Most of those TFs are involved in oxidative stress response as well as lipid metabolism pathways (Gad et al. 2012).

Previous evidence indicated the deleterious effects of high oxygen level-induced oxidative stress and the survival of preimplantation embryos under *in vitro* condition is attributed to the ability of embryos to activate NRF2 signaling pathway (Amin et al. 2014). However, this response could be differentially expressed depending on the sex of embryos, which are transcriptionally, epigenetically as well as metabolically sexual dimorphic (Bermejo-Alvarez et al. 2008; Bermejo-Alvarez et al. 2010; Gomez et al. 2018). These differences could partially arise from X-chromosome inactivation (XCI), which is not entirely completed during early preimplantation stages (Bermejo-Alvarez et al. 2010). However, it could be extended to day 14 (Bermejo-Alvarez et al. 2011) or day 19 (Forde et al. 2016). The incomplete XCI could regulate genes linked to not only sex chromosomes but also autosomal chromosomes (Pérez-Cerezales et al. 2018). For instance, exposure of male and female mouse embryos to heat stress-induced oxidative stress showed an upregulation of glucose-6-phosphate dehydrogenase (G6PD), an X-linked gene related to oxygen radicals scavenging, which emphasize the resistance of female embryos under these circumstances (Pérez-Crespo et al. 2005). Similarly, G6PD was observed in male and female bovine embryos at the morula stage but not at the blastocyst stage exposed to oxidative stress (Iwata et al. 2002), which indicates the superiority of female embryos till morula stage. From this notion, the expression of the NRF2 signaling pathway could be differentially expressed between male and female embryos under oxidative stress (Figure 3.4 and 3.5) and consequently the machinery of antioxidants (Figure 3.7).

The expression of NRF2 is partially involved in delaying differentiation of human embryonic stem cells via regulation of self-renewal and pluripotency-related genes including POU5F1 and NANOG (Jang et al. 2014), which could also be regulated via KLF4 (Chan et al. 2009). Accordingly, those TFs were found to be dysregulated under oxidative stress conditions (Leite et al. 2017). These facts were in agreement with the current findings, which showed an upregulation of KLF4 (Figure 3.4 and 3.6) and subsequently POU5F1 (Figure 3.7) in embryos exposed to oxidative stress. On the other hand, the differentiation is found to be sex-specific due to presence or absence X chromosome inactivation (Schulz et al. 2014). Interestingly, the KLF4 protein was observed to be enriched in trophectoderm rather than inner cell mass (Ozawa et al. 2012),

which indicates the cellular specificity of the TFs. Furthermore, the KLF4 was found to regulate genes involved in endoderm formation including GATA4, which is showed to be upregulated under KLF4 knockdown in mouse embryonic stem cells (Aksoy et al. 2014), which is not compatible with the current findings (Figure 3.7). However, the co-expression of GATA4 with pluripotency-related genes, namely KLF4, POU5F1, SOX2 and c-MYC resulted in impairing the reprogramming of mouse embryonic fibroblast cells (Serrano et al. 2013), which indicates the disturbance of stemness pattern under oxidative stress condition. From another point of view, the expression of GATA4 might be involved in other biological function such as cholesterol biogenesis and transport (Bergeron et al. 2015) as well as steroidogenesis (Bergeron et al. 2015; Schrade et al. 2015) and glycolysis (Schrade et al. 2015), which might overlap with SREBFs family functions.

The exposure of bovine preimplantation embryos to high oxygen tension-induced oxidative stress increased the expression of SREBF1 and lipid metabolism-related genes, which might be regulated via NRF2 (Amin et al. 2014). Exposure of embryos to such high oxygen tension could induce endoplasmic reticulum stress, which in turn increases the cleavage of SREBF2 and subsequently cholesterol accumulation (Colgan et al. 2007). These facts could partially explain the reasons for increasing lipid accumulation in embryos produced *in vitro* (Takahashi et al. 2013). Moreover, the potential release of lipid and lipid-like molecules into extracellular space could be sex-dependent, which was proven by higher releasing of such molecules by male embryos compared to female counterparts (Gomez et al. 2018). This could be also associated with developmental sexual dimorphism (Xu et al. 1992), which corroborates the current results (Figure 3.4).

Cell apoptosis is also supposed to be sex-depended and was remarkably observed in female embryos (Ghys et al. 2016; Oliveira et al. 2016). This phenomenon could be regulated via several pathways including caspase 3 (Carvour et al. 2008; Ghys et al. 2016; Wolf et al. 1999), which revealed to be regulated via E2F1 (Ginsberg 2002; Shelat et al. 2001). This fact indicates the differential expression of E2F1 between male and female embryos (Figure 3.4) to induce apoptosis, which in turn leads to a reduction in total cell number (Ghys et al. 2016; Leite et al. 2017). However, cells might respond to such stress via activation NOTCH1, which in turn activates a cascade of genes to ultimately preventing cellular apoptosis via suppression of the p38 MAPK signaling pathway (Mo et al. 2013). From another point of view, the previous study found the recruitment of NOTCH signaling pathway in mouse trophectoderm (Rayon et al. 2014), which might be

associated with increasing trophectoderm:inner cell mass ratio in bovine female embryos compared to male ones (Siqueira and Hansen 2016). Interestingly, the disturbance in the expression of NRF2 led to disturbing the expression of NOTCH signaling pathway and their downstream genes (Wakabayashi et al. 2015), which indicates the potential regulation of NRF2 to NOTCH signaling pathway. These findings confirm the differential expression of NOTCH1 in the current study under oxidative stress condition (Figure 3.4). Intriguingly, those TFs could be transferred to extracellular space in order to maintain cellular homeostasis or alter the biological functions of neighboring cells.

During the last decades, a series of experiments were conducted to improve *in vitro* embryo culture model. During that, the scientists found that embryo group culture (Kelley and Gardner 2016) and co-culture with somatic cells either in groups (Donnay et al. 1997; Miranda et al. 2016) or solitary (Donnay et al. 1997) boosted the blastocyst development compared to individual culture. This fact argues the presence of mediators that compensate for the absence of the maternal reproductive tract and enhance cell-cell communication. Several mediators are believed to be involved in this communication such as miRNA (Kropp et al. 2014) and extracellular vesicles (EVs) (Mellisho et al. 2017; Qu et al. 2017).

EVs were found in various biological fluids such as milk, plasma, semen (Lawson et al. 2016), follicular fluid (Hung et al. 2015) and uterine fluids (Campoy et al. 2016). Additionally, EVs were also found to be released into the culture media of bovine granulosa cells (Saeed-Zidane et al. 2017) and preimplantation embryos (Pavani et al. 2018). Due to their ability to carry genetic material and metabolites, they are involved in altering cell functions during either physiological (Hung et al. 2017; Valadi et al. 2007) or pathological (An et al. 2015; Anderson et al. 2010) conditions. Moreover, they can transfer the harmful molecules from the cell into extracellular space in order to maintain cellular homeostasis (Takahashi et al. 2017). According to the releasing mechanism of EVs subtypes, apoptotic bodies (ABs) are released directly from the cellular membrane of apoptotic cells (Lawson et al. 2016). However, microvesicles (MVs) are secreted from lived cells via outward budding (Tricarico et al. 2017). Furthermore, exosomes (EXOs) are also released from lived cells via invaginations multivesicular bodies (MVBs) limiting membrane, which is subsequently fused to the cellular membrane and released their content into extracellular space (Pant et al. 2012). Several proteins involved in EXOs biogenesis processes are normally used in identifying the EXOs population including

tetraspanins (CD63, CD81 and CD9), ALIX, flotillin-1 and TSG101 (Colombo et al. 2014; Jia et al. 2017; Mathivanan et al. 2010). Currently, the term of EVs is more preferable rather than exosomes or microvesicles due to the overlapping between subtypes in terms of size (Machtinger et al. 2016; Pavani et al. 2016; Raposo and Stoorvogel 2013). Accordingly, the CD63 was detected in the current study to check the purity of isolated EVs (Figure 3.8A).

EVs contents could be differed depending on the physiological status (Hung et al. 2017; Navakanitworakul et al. 2016), inflammation (Console et al. 2019; Jolly et al. 1975), and stress response (Beninson et al. 2014; Bewicke-Copley et al. 2017; Eldh et al. 2010). For instance, exposure of granulosa cells to oxidative stress resulted in releasing EVs enriched with NRF2 and antioxidant genes (Saeed-Zidane et al. 2017), which is in agreement with this study (Figure 3.10). On the other hand, the comprehensive study of miRNA in endometrial epithelial cells and their released-EVs showed differential expression of some miRNAs between cells and their secretome involved in embryo-maternal communication. The analysis of target genes of those miRNAs revealed their contributions in embryo implantation (Ng et al. 2013), which indicates the selectivity of EVs to their cargo. Furthermore, the number of released-EVs could be increased under stress conditions, as evidenced by increasing released-EVs numbers of granulosa cells in response to H_2O_2-induced oxidative stress (Saeed-Zidane et al. 2017), which could be potentially regulated during their biogenesis.

Endosomal Sorting Complex Required for Transport (ESCRT) machinery is considered the major pathway involved in EXOs biogenesis, which contains four protein complexes; namely, ESCRT-0 influences the clustering of cargo in a ubiquitin-dependent manner, ESCRT-1 and -2 trigger bud formation and ESCRT-3 boosts vesicles separation, as well as associated proteins including AAA ATPase VPS4 involved in the segregation and recycling of the ESCRT machinery (Hessvik and Llorente 2018; Katzmann et al. 2001; Kowal et al. 2014). Supporting to this notion, the depletion of TSG101, which is a member of ESCRT-1 protein complexes, led to compromise the number of released-EXOs in Hela cells. However, the opposite results were observed in the case of VPS4B deficiency (Colombo et al. 2013). Meanwhile, increasing numbers of released-EXOs were observed in the MCF-7 cell line after the silencing of two isoforms of VPS4 (Baietti et al. 2012), which fits well with the present study (Figure 3.9). These contradicting results could indicate that the expression of those genes could be cell-dependent. Screening

studies showed that inhibition of different member of RAB family such as RAB27A, RAB27B, RAB35 and RAB11, which facilitate the fusion to cellular membrane and secretion of EXOs to extracellular space, resulted in reduction of released-EXOs (Blanc and Vidal 2018; Ostrowski et al. 2010; Takahashi et al. 2017; Yeung et al. 2018), which indicate their importance role in EXOs biogenesis and secretion. These results confirm the current findings, which showed an increased level of RAB11 and RAB27A in male embryos under the high oxygen level (Figure 3.9) and consequently increasing the released-EVs (Figure 3.8).

4.2 Conclusion and future perspective

Taken together, in this study, in addition to antioxidant machinery in bovine granulosa cells and preimplantation embryos were found to respond to oxidative stress through the alteration of cross-talking candidate TFs involved in cell apoptosis, lipid biosynthesis, and differentiation. The cellular and extracellular mRNA expression level of TFs as well as NRF2 and their antioxidant downstream genes led to the survival of male embryos compared to female counterparts upon exposure to oxidative stress.

These studies provide basic information to perform further studies that take into account the following points:

- The transcription and post-transcription regulatory mechanisms of the transcription factors during granulosa cells luteinization and preimplantation embryos development

- Reciprocals horizontal transmission of extracellular vesicles cargo between male and female embryos exposed to various environmental stressors in stage dependent-manner and their role in adaptive response as well as development

- Using CRISPR/Cas9-mediated gene editing to investigate the potential role of ESCRT machinery in bovine extracellular vesicles biogenesis and secretion

- Holistic analysis of extracellular vesicles under different environmental stresses in follicular cells and preimplantation embryos

4.3 References

Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG (2006) Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 86:503-512

Aksoy I, Giudice V, Delahaye E, Wianny F, Aubry M, Mure M, Chen J, Jauch R, Bogu GK, Nolden T, Himmelbauer H, Xavier Doss M, Sachinidis A, Schulz H, Hummel O, Martinelli P, Hubner N, Stanton LW, Real FX, Bourillot PY, Savatier P (2014) Klf4 and Klf5 differentially inhibit mesoderm and endoderm differentiation in embryonic stem cells. Nat Commun 5:3719

Alemu TW, Pandey HO, Salilew Wondim D, Gebremedhn S, Neuhof C, Tholen E, Holker M, Schellander K, Tesfaye D (2018) Oxidative and endoplasmic reticulum stress defense mechanisms of bovine granulosa cells exposed to heat stress. Theriogenology 110:130-141

Amin A, Gad A, Salilew-Wondim D, Prastowo S, Held E, Hoelker M, Rings F, Tholen E, Neuhoff C, Looft C, Schellander K, Tesfaye D (2014) Bovine embryo survival under oxidative-stress conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway. Mol Reprod Dev 81:497-513

An T, Qin S, Xu Y, Tang Y, Huang Y, Situ B, Inal JM, Zheng L (2015) Exosomes serve as tumour markers for personalized diagnostics owing to their important role in cancer metastasis. J Extracell Vesicles 4:27522

Anderson HC, Mulhall D, Garimella R (2010) Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. Lab Invest 90:1549-1557

Andersson ER, Sandberg R, Lendahl U (2011) Notch signaling: simplicity in design, versatility in function. Development 138:3593-3612

Andreu-Vieyra C, Chen R, Matzuk MM (2008) Conditional deletion of the retinoblastoma (Rb) gene in ovarian granulosa cells leads to premature ovarian failure. Mol Endocrinol 22:2141-2161

Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, Ivarsson Y, Depoortere F, Coomans C, Vermeiren E, Zimmermann P, David G (2012) Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol 14:677-685

Beninson LA, Brown PN, Loughridge AB, Saludes JP, Maslanik T, Hills AK, Woodworth T, Craig W, Yin H, Fleshner M (2014) Acute stressor exposure

modifies plasma exosome-associated heat shock protein 72 (Hsp72) and microRNA (miR-142-5p and miR-203). PLoS One 9:e108748

Bergeron F, Nadeau G, Viger RS (2015) GATA4 knockdown in MA-10 Leydig cells identifies multiple target genes in the steroidogenic pathway. Reproduction 149:245-257

Bermejo-Alvarez P, Rizos D, Lonergan P, Gutierrez-Adan A (2011) Transcriptional sexual dimorphism in elongating bovine embryos: implications for XCI and sex determination genes. Reproduction 141:801-808

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2008) Epigenetic differences between male and female bovine blastocysts produced in vitro. Physiol Genomics 32:264-272

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2010) Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proc Natl Acad Sci U S A 107:3394-3399

Bewicke-Copley F, Mulcahy LA, Jacobs LA, Samuel P, Akbar N, Pink RC, Carter DRF (2017) Extracellular vesicles released following heat stress induce bystander effect in unstressed populations. J Extracell Vesicles 6:1340746

Blanc L, Vidal M (2018) New insights into the function of Rab GTPases in the context of exosomal secretion. Small GTPases 9:95-106

Bray SJ (2006) Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7:678-689

Cagnone G, Sirard MA (2016) The embryonic stress response to in vitro culture: insight from genomic analysis. Reproduction 152:R247-R261

Campoy I, Lanau L, Altadill T, Sequeiros T, Cabrera S, Cubo-Abert M, Perez-Benavente A, Garcia A, Borros S, Santamaria A, Ponce J, Matias-Guiu X, Reventos J, Gil-Moreno A, Rigau M, Colas E (2016) Exosome-like vesicles in uterine aspirates: a comparison of ultracentrifugation-based isolation protocols. J Transl Med 14:180

Carletti MZ, Christenson LK (2009) Rapid effects of LH on gene expression in the mural granulosa cells of mouse periovulatory follicles. Reproduction 137:843-855

Carvour M, Song C, Kaul S, Anantharam V, Kanthasamy A, Kanthasamy A (2008) Chronic low-dose oxidative stress induces caspase-3-dependent PKCdelta

proteolytic activation and apoptosis in a cell culture model of dopaminergic neurodegeneration. Ann N Y Acad Sci 1139:197-205

Chan KK, Zhang J, Chia NY, Chan YS, Sim HS, Tan KS, Oh SK, Ng HH, Choo AB (2009) KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells. Stem Cells 27:2114-2125

Chen Z, Wen L, Martin M, Hsu CY, Fang L, Lin FM, Lin TY, Geary MJ, Geary GG, Zhao Y, Johnson DA, Chen JW, Lin SJ, Chien S, Huang HD, Miller YI, Huang PH, Shyy JY (2015) Oxidative stress activates endothelial innate immunity via sterol regulatory element binding protein 2 (SREBP2) transactivation of microRNA-92a. Circulation 131:805-814

Choi H, Roh J (2018) Role of Klf4 in the Regulation of Apoptosis and Cell Cycle in Rat Granulosa Cells during the Periovulatory Period. Int J Mol Sci 20:

Choi H, Ryu KY, Roh J (2019) Kruppel-like factor 4 plays a role in the luteal transition in steroidogenesis by down-regulating Cyp19A1 expression. Am J Physiol Endocrinol Metab

Chu PW, Wang YP, Chen IC, Pan HM, Wu GJ (2011) Notch 1 signaling pathway effect on implantation competency. Fertil Steril 96:1225-1229

Colgan SM, Tang D, Werstuck GH, Austin RC (2007) Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2. Int J Biochem Cell Biol 39:1843-1851

Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, Manel N, Moita LF, Thery C, Raposo G (2013) Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci 126:5553-5565

Colombo M, Raposo G, Thery C (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 30:255-289

Cong X, Zhang Q, Li H, Jiang Z, Cao R, Gao S, Tian W (2017) Puerarin ameliorates heat stress-induced oxidative damage and apoptosis in bovine Sertoli cells by suppressing ROS production and upregulating Hsp72 expression. Theriogenology 88:215-227

Console L, Scalise M, Indiveri C (2019) Exosomes in inflammation and role as biomarkers. Clin Chim Acta 488:165-171

Craig HJB, Stachowicz K, Black M, Parry M, Burke CR, Meier S, Amer PR (2018) Genotype by environment interactions in fertility traits in New Zealand dairy cows. J Dairy Sci 101:10991-11003

Crespo D, Bonnet E, Roher N, MacKenzie SA, Krasnov A, Goetz FW, Bobe J, Planas JV (2010) Cellular and molecular evidence for a role of tumor necrosis factor alpha in the ovulatory mechanism of trout. Reprod Biol Endocrinol 8:34

da Silveira WA, Palma PVB, Sicchieri RD, Villacis RAR, Mandarano LRM, Oliveira TMG, Antonio HMR, Andrade JM, Muglia VF, Rogatto SR, Theillet C, du Manoir S, Tiezzi DG (2017) Transcription factor networks derived from breast cancer stem cells control the immune response in the basal subtype. Sci Rep 7:2851

Daemen S, Kutmon M, Evelo CT (2013) A pathway approach to investigate the function and regulation of SREBPs. Genes Nutr 8:289-300

Delidow BC, Lynch JP, White BA, Peluso JJ (1992) Regulation of proto-oncogene expression and deoxyribonucleic acid synthesis in granulosa cells of perifused immature rat ovaries. Biol Reprod 47:428-435

Dias FC, Khan MI, Sirard MA, Adams GP, Singh J (2013) Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. Reproduction 146:181-191

Dick FA, Dyson N (2003) pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. Mol Cell 12:639-649

Donnay I, Van Langendonckt A, Auquier P, Grisart B, Vansteenbrugge A, Massip A, Dessy F (1997) Effects of co-culture and embryo number on the in vitro development of bovine embryos. Theriogenology 47:1549-1561

Douville G, Sirard MA (2014) Changes in granulosa cells gene expression associated with growth, plateau and atretic phases in medium bovine follicles. J Ovarian Res 7:50

Duffy DM, Ko C, Jo M, Brannstrom M, Curry TE (2019) Ovulation: parallels with inflammatory processes. Endocr Rev 40:369-416

Dzafic E, Stimpfel M, Virant-Klun I (2013) Plasticity of granulosa cells: on the crossroad of stemness and transdifferentiation potential. J Assist Reprod Genet 30:1255-1261

Eldh M, Ekstrom K, Valadi H, Sjostrand M, Olsson B, Jernas M, Lotvall J (2010) Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. PLoS One 5:e15353

Fang DL, Wan Y, Shen W, Cao J, Sun ZX, Yu HH, Zhang Q, Cheng WH, Chen J, Ning B (2013) Endoplasmic reticulum stress leads to lipid accumulation through upregulation of SREBP-1c in normal hepatic and hepatoma cells. Mol Cell Biochem 381:127-137

Fischer B, Bavister BD (1993) Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil 99:673-679

Forde N, Maillo V, O'Gaora P, Simintiras CA, Sturmey RG, Ealy AD, Spencer TE, Gutierrez-Adan A, Rizos D, Lonergan P (2016) Sexually dimorphic gene expression in bovine conceptuses at the initiation of implantation. Biol Reprod 95:92

Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D (2012) Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. Biol Reprod 87:100

Garmey JC, Guthrie HD, Garrett WM, Stoler MH, Veldhuis JD (2000) Localization and expression of low-density lipoprotein receptor, steroidogenic acute regulatory protein, cytochrome P450 side-chain cleavage and P450 17-alpha-hydroxylase/C17-20 lyase in developing swine follicles: in situ molecular hybridization and immunocytochemical studies. Mol Cell Endocrinol 170:57-65

Ghys E, Dallemagne M, De Troy D, Sauvegarde C, Errachid A, Donnay I (2016) Female bovine blastocysts are more prone to apoptosis than male ones. Theriogenology 85:591-600

Gilbert I, Robert C, Dieleman S, Blondin P, Sirard MA (2011) Transcriptional effect of the LH surge in bovine granulosa cells during the peri-ovulation period. Reproduction 141:193-205

Ginsberg D (2002) E2F1 pathways to apoptosis. FEBS Lett 529:122-125

Gomez E, Carrocera S, Martin D, Herrero P, Canela N, Munoz M (2018) Differential release of cell-signaling metabolites by male and female bovine embryos cultured in vitro. Theriogenology 114:180-184

Griffiths B, Lewis CA, Bensaad K, Ros S, Zhang Q, Ferber EC, Konisti S, Peck B, Miess H, East P, Wakelam M, Harris AL, Schulze A (2013) Sterol regulatory element binding protein-dependent regulation of lipid synthesis supports cell survival and tumor growth. Cancer Metab 1:3

Guerrero-Netro HM, Chorfi Y, Price CA (2015) Effects of the mycotoxin deoxynivalenol on steroidogenesis and apoptosis in granulosa cells. Reproduction 149:555-561

Hatzirodos N, Hummitzsch K, Irving-Rodgers HF, Harland ML, Morris SE, Rodgers RJ (2014a) Transcriptome profiling of granulosa cells from bovine ovarian follicles during atresia. BMC Genomics 15:40

Hatzirodos N, Irving-Rodgers HF, Hummitzsch K, Rodgers RJ (2014b) Transcriptome profiling of the theca interna from bovine ovarian follicles during atresia. PLoS One 9:e99706

Hellweg CE, Spitta LF, Henschenmacher B, Diegeler S, Baumstark-Khan C (2016) Transcription Factors in the Cellular Response to Charged Particle Exposure. Front Oncol 6:61

Hennet ML, Yu HY, Combelles CM (2013) Follicular fluid hydrogen peroxide and lipid hydroperoxide in bovine antral follicles of various size, atresia, and dominance status. J Assist Reprod Genet 30:333-340

Hessvik NP, Llorente A (2018) Current knowledge on exosome biogenesis and release. Cell Mol Life Sci 75:193-208

Homma S, Ishii Y, Morishima Y, Yamadori T, Matsuno Y, Haraguchi N, Kikuchi N, Satoh H, Sakamoto T, Hizawa N, Itoh K, Yamamoto M (2009) Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. Clin Cancer Res 15:3423-3432

Hsieh MC, Das D, Sambandam N, Zhang MQ, Nahle Z (2008) Regulation of the PDK4 isozyme by the Rb-E2F1 complex. J Biol Chem 283:27410-27417

Hsueh AJ, Jones PB (1981) Extrapituitary actions of gonadotropin-releasing hormone. Endocr Rev 2:437-461

Huang N, Yu Y, Qiao J (2017) Dual role for the unfolded protein response in the ovary: adaption and apoptosis. Protein Cell 8:14-24

Hung WT, Hong X, Christenson LK, McGinnis LK (2015) Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biol Reprod 93:117

Hung WT, Navakanitworakul R, Khan T, Zhang P, Davis JS, McGinnis LK, Christenson LK (2017) Stage-specific follicular extracellular vesicle uptake and regulation of bovine granulosa cell proliferation. Biol Reprod 97:644-655

Hussein MR (2005) Apoptosis in the ovary: molecular mechanisms. Hum Reprod Update 11:162-177

Iaquinta PJ, Lees JA (2007) Life and death decisions by the E2F transcription factors. Curr Opin Cell Biol 19:649-657

Iwata H, Kimura K, Hashimoto S, Ohta M, Tominaga K, Minami N (2002) Role of G6PD activity on sex ratio and developmental competence of bovine embryos under oxidative stress. J Reprod Develop 48:447–453

Jang J, Wang Y, Kim HS, Lalli MA, Kosik KS (2014) Nrf2, a regulator of the proteasome, controls self-renewal and pluripotency in human embryonic stem cells. Stem Cells 32:2616-2625

Jia Y, Chen Y, Wang Q, Jayasinghe U, Luo X, Wei Q, Wang J, Xiong H, Chen C, Xu B, Hu W, Wang L, Zhao W, Zhou J (2017) Exosome: emerging biomarker in breast cancer. Oncotarget 8:41717-41733

Jolly RD, Thompson KG, Winchester BG (1975) Bovine mannosidosis--a model lysosomal storage disease. Birth Defects Orig Artic Ser 11:273-278

Kaczynski J, Cook T, Urrutia R (2003) Sp1- and Kruppel-like transcription factors. Genome Biol 4:206

Kamisako T, Tanaka Y, Kishino Y, Ikeda T, Yamamoto K, Masuda S, Ogawa H (2014) Role of Nrf2 in the alteration of cholesterol and bile acid metabolism-related gene expression by dietary cholesterol in high fat-fed mice. J Clin Biochem Nutr 54:90-94

Katzmann DJ, Babst M, Emr SD (2001) Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell 106:145-155

Kelley RL, Gardner DK (2016) Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. Reprod Biomed Online 33:537-549

Kim JK, Kang KA, Ryu YS, Piao MJ, Han X, Oh MC, Boo SJ, Jeong SU, Jeong YJ, Chae S, Na SY, Hyun JW (2016) Induction of endoplasmic reticulum stress via reactive

oxygen species mediated by luteolin in melanoma cells. Anticancer Res 36:2281-2289

Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137:216-233

Kowal J, Tkach M, Thery C (2014) Biogenesis and secretion of exosomes. Curr Opin Cell Biol 29:116-125

Kropp J, Salih SM, Khatib H (2014) Expression of microRNAs in bovine and human pre-implantation embryo culture media. Front Genet 5:91

Lai Q, Xiang W, Li Q, Zhang H, Li Y, Zhu G, Xiong C, Jin L (2018) Oxidative stress in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with polycystic ovary syndrome. Front Med 12:518-524

LaVoie HA (2017) Transcriptional control of genes mediating ovarian follicular growth, differentiation, and steroidogenesis in pigs. Mol Reprod Dev 84:788-801

Lawson C, Vicencio JM, Yellon DM, Davidson SM (2016) Microvesicles and exosomes: new players in metabolic and cardiovascular disease. J Endocrinol 228:R57-71

Leite RF, Annes K, Ispada J, de Lima CB, Dos Santos EC, Fontes PK, Nogueira MFG, Milazzotto MP (2017) Oxidative stress alters the profile of transcription factors related to early development on in vitro produced embryos. Oxid Med Cell Longev 2017:1502489

Lin P, Yang Y, Li X, Chen F, Cui C, Hu L, Li Q, Liu W, Jin Y (2012) Endoplasmic reticulum stress is involved in granulosa cell apoptosis during follicular atresia in goat ovaries. Mol Reprod Dev 79:423-432

Liu Z, Rudd MD, Hernandez-Gonzalez I, Gonzalez-Robayna I, Fan HY, Zeleznik AJ, Richards JS (2009) FSH and FOXO1 regulate genes in the sterol/steroid and lipid biosynthetic pathways in granulosa cells. Mol Endocrinol 23:649-661

Machtinger R, Laurent LC, Baccarelli AA (2016) Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. Hum Reprod Update 22:182-193

Malhotra JD, Miao H, Zhang K, Wolfson A, Pennathur S, Pipe SW, Kaufman RJ (2008) Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proc Natl Acad Sci U S A 105:18525-18530

Mathivanan S, Ji H, Simpson RJ (2010) Exosomes: extracellular organelles important in intercellular communication. J Proteomics 73:1907-1920

Mellisho EA, Velasquez AE, Nunez MJ, Cabezas JG, Cueto JA, Fader C, Castro FO, Rodriguez-Alvarez L (2017) Identification and characteristics of extracellular vesicles from bovine blastocysts produced in vitro. PLoS One 12:e0178306

Miranda MS, Nascimento HS, Costa MP, Costa NN, Brito KN, Lopes CT, Santos SS, Cordeiro MS, Ohashi OM (2016) Increasing of blastocyst rate and gene expression in co-culture of bovine embryos with adult adipose tissue-derived mesenchymal stem cells. J Assist Reprod Genet 33:1395-1403

Mo JS, Yoon JH, Ann EJ, Ahn JS, Baek HJ, Lee HJ, Kim SH, Kim YD, Kim MY, Park HS (2013) Notch1 modulates oxidative stress induced cell death through suppression of apoptosis signal-regulating kinase 1. Proc Natl Acad Sci U S A 110:6865-6870

Montilla SI, Johnson TP, Pearce SC, Gardan-Salmon D, Gabler NK, Ross JW, Rhoads RP, Baumgard LH, Lonergan SM, Selsby JT (2014) Heat stress causes oxidative stress but not inflammatory signaling in porcine skeletal muscle. Temperature (Austin) 1:42-50

Naji M, Nekoonam S, Aleyasin A, Arefian E, Mahdian R, Azizi E, Shabani Nashtaei M, Amidi F (2018) Expression of miR-15a, miR-145, and miR-182 in granulosa-lutein cells, follicular fluid, and serum of women with polycystic ovary syndrome (PCOS). Arch Gynecol Obstet 297:221-231

Natesampillai S, Kerkvliet J, Leung PC, Veldhuis JD (2008) Regulation of Kruppel-like factor 4, 9, and 13 genes and the steroidogenic genes LDLR, StAR, and CYP11A in ovarian granulosa cells. Am J Physiol Endocrinol Metab 294:E385-391

Navakanitworakul R, Hung WT, Gunewardena S, Davis JS, Chotigeat W, Christenson LK (2016) Characterization and small RNA content of extracellular vesicles in follicular fluid of developing bovine antral follicles. Sci Rep 6:25486

Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL, Salamonsen LA (2013) Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. PLoS One 8:e58502

Oliveira CS, Saraiva NZ, de Lima MR, Oliveira LZ, Serapiao RV, Garcia JM, Borges CA, Camargo LS (2016) Cell death is involved in sexual dimorphism during preimplantation development. Mech Dev 139:42-50

Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, Moita CF, Schauer K, Hume AN, Freitas RP, Goud B, Benaroch P, Hacohen N, Fukuda M, Desnos

C, Seabra MC, Darchen F, Amigorena S, Moita LF, Thery C (2010) Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol 12:19-30; sup pp 11-13

Ozawa M, Sakatani M, Yao J, Shanker S, Yu F, Yamashita R, Wakabayashi S, Nakai K, Dobbs KB, Sudano MJ, Farmerie WG, Hansen PJ (2012) Global gene expression of the inner cell mass and trophectoderm of the bovine blastocyst. BMC Dev Biol 12:33

Palaniappan M, Menon KM (2009) Regulation of sterol regulatory element-binding transcription factor 1a by human chorionic gonadotropin and insulin in cultured rat theca-interstitial cells. Biol Reprod 81:284-292

Pant S, Hilton H, Burczynski ME (2012) The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. Biochem Pharmacol 83:1484-1494

Parborell F, Irusta G, Vitale A, Gonzalez O, Pecci A, Tesone M (2005) Gonadotropin-releasing hormone antagonist antide inhibits apoptosis of preovulatory follicle cells in rat ovary. Biol Reprod 72:659-666

Pavani KC, Alminana C, Wydooghe E, Catteeuw M, Ramirez MA, Mermillod P, Rizos D, Van Soom A (2016) Emerging role of extracellular vesicles in communication of preimplantation embryos in vitro. Reprod Fertil Dev 29:66-83

Pavani KC, Hendrix A, Van Den Broeck W, Couck L, Szymanska K, Lin X, De Koster J, Van Soom A, Leemans B (2018) Isolation and characterization of functionally active extracellular vesicles from culture medium conditioned by bovine embryos in vitro. Int J Mol Sci 20:

Pérez-Cerezales S, Ramos-Ibeas P, Rizos D, Lonergan P, Bermejo-Alvarez P, Gutiérrez-Adán A (2018) Early sex-dependent differences in response to environmental stress. Reproduction 155:R39-R51

Pérez-Crespo M, Ramírez MA, Fernández-González R, Rizos D, Lonergan P, Pintado B, Gutiérrez-Adán A (2005) Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. Mol Reprod Dev 72:502-510

Prasasya RD, Mayo KE (2018) Notch signaling regulates differentiation and steroidogenesis in female mouse ovarian granulosa cells. Endocrinology 159:184-198

Preston GA, Lyon TT, Yin Y, Lang JE, Solomon G, Annab L, Srinivasan DG, Alcorta DA, Barrett JC (1996) Induction of apoptosis by c-Fos protein. Mol Cell Biol 16:211-218

Putman AK, Brown JL, Gandy JC, Wisnieski L, Sordillo LM (2018) Changes in biomarkers of nutrient metabolism, inflammation, and oxidative stress in dairy cows during the transition into the early dry period. J Dairy Sci 101:9350-9359

Qu P, Qing S, Liu R, Qin H, Wang W, Qiao F, Ge H, Liu J, Zhang Y, Cui W, Wang Y (2017) Effects of embryo-derived exosomes on the development of bovine cloned embryos. PLoS One 12:e0174535

Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 200:373-383

Rayon T, Menchero S, Nieto A, Xenopoulos P, Crespo M, Cockburn K, Canon S, Sasaki H, Hadjantonakis AK, de la Pompa JL, Rossant J, Manzanares M (2014) Notch and hippo converge on Cdx2 to specify the trophectoderm lineage in the mouse blastocyst. Dev Cell 30:410-422

Regan SLP, Knight PG, Yovich JL, Leung Y, Arfuso F, Dharmarajan A (2018) Granulosa cell apoptosis in the ovarian follicle-a changing view. Front Endocrinol (Lausanne) 9:61

Rong S, Cortes VA, Rashid S, Anderson NN, McDonald JG, Liang G, Moon YA, Hammer RE, Horton JD (2017) Expression of SREBP-1c requires SREBP-2-mediated generation of a sterol ligand for LXR in livers of mice. Elife 6:

Saeed-Zidane M, Linden L, Salilew-Wondim D, Held E, Neuhoff C, Tholen E, Hoelker M, Schellander K, Tesfaye D (2017) Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress. PLoS One 12:e0187569

Sakatani M (2017) Effects of heat stress on bovine preimplantation embryos produced in vitro. J Reprod Dev 63:347-352

Schrade A, Kyronlahti A, Akinrinade O, Pihlajoki M, Hakkinen M, Fischer S, Alastalo TP, Velagapudi V, Toppari J, Wilson DB, Heikinheimo M (2015) GATA4 is a key regulator of steroidogenesis and glycolysis in mouse leydig cells. Endocrinology 156:1860-1872

Schulz EG, Meisig J, Nakamura T, Okamoto I, Sieber A, Picard C, Borensztein M, Saitou M, Bluthgen N, Heard E (2014) The two active X chromosomes in female ESCs

block exit from the pluripotent state by modulating the ESC signaling network. Cell Stem Cell 14:203-216

Serrano F, Calatayud CF, Blazquez M, Torres J, Castell JV, Bort R (2013) Gata4 blocks somatic cell reprogramming by directly repressing Nanog. Stem Cells 31:71-82

Sharma D, Ghai S, Singh D (2009) Different promoter usage for CYP19 gene expression in buffalo ovary and placenta. Gen Comp Endocrinol 162:319-328

Sharma S, Sarkar J, Haldar C, Sinha S (2014) Melatonin reverses Fas, E2F-1 and endoplasmic reticulum stress mediated apoptosis and dysregulation of autophagy induced by the herbicide atrazine in murine splenocytes. PLoS One 9:e108602

Shelat HS, Liu TJ, Hickman-Bick DL, Barnhart MK, Vida T, Dillard PM, Willerson JT, Zoldhelyi P (2001) Growth suppression of human coronary vascular smooth muscle cells by gene transfer of the transcription factor E2F-1. Circulation 103:407-414

Shen M, Lin F, Zhang J, Tang Y, Chen WK, Liu H (2012) Involvement of the up-regulated FoxO1 expression in follicular granulosa cell apoptosis induced by oxidative stress. J Biol Chem 287:25727-25740

Shi H, Guo Y, Liu Y, Shi B, Guo X, Jin L, Yan S (2016) The in vitro effect of lipopolysaccharide on proliferation, inflammatory factors and antioxidant enzyme activity in bovine mammary epithelial cells. Anim Nutr 2:99-104

Shimano H (2001) Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. Prog Lipid Res 40:439-452

Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N (2011) Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci U S A 108:1462-1467

Sindan N, Bhandari A, Zhao Y, Lu X, Lv J (2018) Expression and localization of nuclear factor erythroid 2-related factor 2 in the ovarian tissues of mice at different ages. Exp Ther Med 16:3546-3552

Siqueira LG, Hansen PJ (2016) Sex differences in response of the bovine embryo to colony-stimulating factor 2. Reproduction 152:645-654

Sohel MMH, Akyuz B, Konca Y, Arslan K, Sariozkan S, Cinar MU (2019) Oxidative stress modulates the expression of apoptosis-associated microRNAs in bovine granulosa cells in vitro. Cell Tissue Res

Sohel MMH, Amin A, Prastowo S, Linares-Otoya L, Hoelker M, Schellander K, Tesfaye D (2018) Sulforaphane protects granulosa cells against oxidative stress via activation of NRF2-ARE pathway. Cell Tissue Res 374:629-641

Taguchi K, Motohashi H, Yamamoto M (2011) Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. Genes Cells 16:123-140

Takahashi A, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S, Takasugi M, Watanabe S, Kanemaki MT, Obuse C, Hara E (2017) Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. Nat Commun 8:15287

Takahashi M (2012) Oxidative stress and redox regulation on in vitro development of mammalian embryos. J Reprod Dev 58:1-9

Takahashi T, Inaba Y, Somfai T, Kaneda M, Geshi M, Nagai T, Manabe N (2013) Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro. Reprod Fertil Dev 25:589-599

Tanabe M, Tamura H, Taketani T, Okada M, Lee L, Tamura I, Maekawa R, Asada H, Yamagata Y, Sugino N (2015) Melatonin protects the integrity of granulosa cells by reducing oxidative stress in nuclei, mitochondria, and plasma membranes in mice. J Reprod Dev 61:35-41

Tilly JL (1996) Apoptosis and ovarian function. Rev Reprod 1:162-172

Tricarico C, Clancy J, D'Souza-Schorey C (2017) Biology and biogenesis of shed microvesicles. Small GTPases 8:220-232

Trombly DJ, Woodruff TK, Mayo KE (2009) Suppression of Notch signaling in the neonatal mouse ovary decreases primordial follicle formation. Endocrinology 150:1014-1024

Urtasun R, Cubero FJ, Nieto N (2012) Oxidative stress modulates KLF6$_{Full}$ and its splice variants. Alcohol Clin Exp Res 36:1851-1862

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654-659

Vanorny DA, Prasasya RD, Chalpe AJ, Kilen SM, Mayo KE (2014) Notch signaling regulates ovarian follicle formation and coordinates follicular growth. Mol Endocrinol 28:499-511

Vanselow J, Vernunft A, Koczan D, Spitschak M, Kuhla B (2016) Exposure of lactating dairy cows to acute pre-ovulatory heat stress affects granulosa cell-specific gene expression profiles in dominant follicles. PLoS One 11:e0160600

Vaux DL, Strasser A (1996) The molecular biology of apoptosis. Proc Natl Acad Sci U S A 93:2239-2244

Viatour P, Ehmer U, Saddic LA, Dorrell C, Andersen JB, Lin C, Zmoos AF, Mazur PK, Schaffer BE, Ostermeier A, Vogel H, Sylvester KG, Thorgeirsson SS, Grompe M, Sage J (2011) Notch signaling inhibits hepatocellular carcinoma following inactivation of the RB pathway. J Exp Med 208:1963-1976

Wakabayashi N, Chartoumpekis DV, Kensler TW (2015) Crosstalk between Nrf2 and Notch signaling. Free Radic Biol Med 88:158-167

Wale PL, Gardner DK (2016) The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. Hum Reprod Update 22:2-22

Wolf BB, Schuler M, Echeverri F, Green DR (1999) Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. J Biol Chem 274:30651-30656

Wu Y, Lin J, Li X, Han B, Wang L, Liu M, Huang J (2017) Transcriptome profile of one-month-old lambs' granulosa cells after superstimulation. Asian-Australas J Anim Sci 30:20-33

Xu J, Gridley T (2013) Notch2 is required in somatic cells for breakdown of ovarian germ-cell nests and formation of primordial follicles. BMC Biol 11:13

Xu KP, Yadav BR, King WA, Betteridge KJ (1992) Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. Mol Reprod Dev 31:249-252

Xu L, Sun H, Zhang M, Jiang Y, Zhang C, Zhou J, Ding L, Hu Y, Yan G (2017) MicroRNA-145 protects follicular granulosa cells against oxidative stress-induced apoptosis by targeting Kruppel-like factor 4. Mol Cell Endocrinol 452:138-147

Yamamoto Y, Kuwahara A, Taniguchi Y, Yamasaki M, Tanaka Y, Mukai Y, Yamashita M, Matsuzaki T, Yasui T, Irahara M (2015) Tumor necrosis factor alpha inhibits ovulation and induces granulosa cell death in rat ovaries. Reprod Med Biol 14:107-115

Yeung V, Webber JP, Dunlop EA, Morgan H, Hutton J, Gurney M, Jones E, Falcon-Perez J, Tabi Z, Errington R, Clayton A (2018) Rab35-dependent extracellular nanovesicles are required for induction of tumour supporting stroma. Nanoscale 10:8547-8559

Yin Y, Wang S, Sun Y, Matt Y, Colburn NH, Shu Y, Han X (2009) JNK/AP-1 pathway is involved in tumor necrosis factor-alpha induced expression of vascular endothelial growth factor in MCF7 cells. Biomed Pharmacother 63:429-435

Zhao Q, Mao A, Yan J, Sun C, Di C, Zhou X, Li H, Guo R, Zhang H (2016) Downregulation of Nrf2 promotes radiation-induced apoptosis through Nrf2 mediated Notch signaling in non-small cell lung cancer cells. Int J Oncol 48:765-773

Zucker SN, Fink EE, Bagati A, Mannava S, Bianchi-Smiraglia A, Bogner PN, Wawrzyniak JA, Foley C, Leonova KI, Grimm MJ, Moparthy K, Ionov Y, Wang J, Liu S, Sexton S, Kandel ES, Bakin AV, Zhang Y, Kaminski N, Segal BH, Nikiforov MA (2014) Nrf2 amplifies oxidative stress via induction of Klf9. Mol Cell 53:916-928

5. Acknowledgments

First and foremost, all praise to Almighty Allah, the most compassionate, the most merciful, who blessed as well as giving me knowledge, ability, power and a great help to successfully finish my PhD thesis. Without his blessings, this achievement would not have been possible.

As in life, collaboration leads to best outcomes. So, I definitely did not reach here on my own. I would like to acknowledge all people, who participate during the journey of my PhD study and making it possible.

At the outset, I would like to express my deepest sense of sincere gratitude to my supervisor, Prof. Dr. Karl Schellander, who had many rightful claims in shaping up my present research work. I am really so grateful for invaluable support, guidance and enthusiastic supervision during my PhD journey. Also, I express my deepest thanks to you for all your commitments- for giving me an opportunity to develop my interest in the field of molecular genetics. I am so proud to be his student and a member of his research team, above all for your molar support and his patience throughout my research study. I would also like to thank Prof. Dr. Helga Sauerwein for her willingness to be my second supervisor, valuable advice and evaluation of my PhD work. My sincere thanks also go to Prof. Dr. Jens Léon and Prof. Dr. Andreas Schieber for their scientific evaluation of this work and defense.

My deepest thanks also go to PD Dr. Dawit Tesfaye, for his constant support and scientific brilliance were decisive in guiding this work. I really enjoyed the thoughtful discussions during our regular meetings that enhanced my interest and knowledge in the area of molecular genetics. I really appreciate his great help in meticulous appraisal of manuscripts, presentations and thesis drafts. I am also deeply grateful to Dr. Ernst Tholen for his helpful suggestions, support and comments during our regular seminars.

My sincere thanks go to Dr. Michael Höelker, Dr. Eva Held and Ms. Franka Rings, for supplying me valuable samples, which are necessary to accomplish my PhD work. I gratefully acknowledge Dr. Dessie Salilew-Wondim for his help, support and manuscript

revision. My deepest thanks go also to Dr. Christiane Neuhoff, for nice help in the German version of the abstract (Zusammenfassung).

I take this opportunity to express my sincere thanks to all my colleagues at the Institute of Animal Science for helping and supporting me in one way or another to successfully accomplish this task, especially; Dr. Maren Pröll, Dr. Julia Welzenbach, Dr. Ahmed Amin, Dr. Ahmed Gad, Dr. Sigit Prastowo, Dr. Rui Zhang, Dr. Qin Yang, Dr. Samuel Etay, Ms. Ester Heuss, Ms. Ines Brinke, Dr. Lucia Matinez-Fresneda, Ms. Katharina Roth, Ms. Hoda Aglan, Dr. Tsige Hagos, Mr. Mikhael Poirier, Mr. Haiko Hofmann, Mr. Dennis Miskel, Dr. Mohamed Bozlur Rahman, Dr. Aminul Islam, Dr. Eryk Andreas, Dr. Sharmin Aqter Rony, Ms. Anna Leechr, Ms. Fenja Piaskowy and Dr. Hari Om Pandey. I would like to express my deep and sincere gratitude to Prof. Dr. Karl-Heinz Südekum and nutrition group members for their support. I would also like to express my thanks to Ms. Renate Kicker for providing her heartfelt support with her smiley face.

My sincerely grateful to Ms. Helga Brodeßer Ms. Birgit Koch-Fabritius, Ms. Stephanie Fuchs and Ms. Nadine Leyer, Mr. Heinz Björnsen for their laboratory help and kind support and nice social memories. I would like to thank all administrative members of the Institute of Animal Science, especially, Ms. Bianca Peters, Mr. Peter Müller, and Mr. Stephan Knauf for their great help, support facilitate things. My thanks to all present and former Azubis, Ms. Luara Kück, Ms. Daria Henseler, Ms. Julia Lindlar, Mr. Klian Geyer and Mr. Michel Posanki. I acknowledge the contribution of Prof. Dr. Andreas Meyer and Dr. Stefanie Müller-Schüssele at Chemical Signalling, Institute of Crop Science and Resource Conservation (INRES), University of Bonn for helping me during the use of the confocal microscope. I would especially like to thank Prof. Joachim Hamacher at Department of Plant Disease and Plant Protection, INRES, University of Bonn for providing me electron microscope. I am also grateful to Dr. Reinhard Bauer at Life and Medical Sciences Institute (LIMES) of the University of Bonn for facilitating me ultracentrifugation. I would like to express my gratitude to Prof. Dr. Elke Pogge von Strandmann and her colleague Maximiliane Schuldner at Innate Immunity Group, Department of Internal Medicine I, University Hospital Cologne for their extraordinary support during nanoparticles analysis.

acknowledging my gratitude to Dr. Fayza Omran for her constant support and encouragement during my study. I would like to express my special thanks of gratitude to my late supervisor Prof. Dr. Yassein Hafez, who was supporting and advising me from the first time I intended to do my PhD abroad. I thank him with all my heart and I am praying for him to be in paradise.

Most importantly, my acknowledgment would be incomplete without thanking the biggest source of my strength, my family. I cannot find suitable words to thank **my parents** enough for all the support and love they have given me. I would like to express my deepest thanks to them; whose love, encouragement, moral support, personal attention and guidance are with me in whatever I pursue. The blessings and prayers of my parents and the love and care of my sisters Marwa and Fatma have all made a tremendous contribution in helping me reach this stage in my life. I thank them for putting up with me in difficult moments where I felt stumped and for goading me on to follow my dream of getting this degree. This would not have been possible without their unwavering and unselfish love and support that they are given to me at all times. I also express my deep sense of thankfulness to my late uncle Saad Ali Farag and his son Mohamed as well as My late Aunt Mahasen Taqi, who were believing in me. I know they are up there, listening, watching over me and sending me her blessings constantly. I would also like to express my gratitude and appreciation to my Uncles, Aunts and my relatives for supporting and encouraging me.

Last but not least, I am indebted and grateful to everyone supporting and motivating me during my either PhD or life journey. I would like to apologize for everyone I did not give him/her suitable words for thanking because I did not find those appropriate words in the English dictionary that give him/her what they deserve. I also apologize for everyone that I did not mention him/her with the name due to the limited space, which could need a book.

6. Research publications

a) Peer-reviewed published articles

- **Mohamed Omar Taqi**, Mohammed Saeed-Zidane, Samuel Gebremedhn, Dessie Salilew-Wondim, Omar Khdrawy, Franca Rings, Christiane Neuhoff, Michael Hoelker, Karl Schellander, Dawit Tesfaye (2019). Sexual dimorphic expression and release of transcription factors in bovine embryos exposed to oxidative stress. Mol. Reprod. Dev., DOI:10.1002/mrd.23272

- Omar Khadrawy, Samuel Gebremedhn, Dessie Salilew-Wondim, **Mohamed Omar Taqi**, Christiane Neuhoff, Ernst Tholen, Michael Hoelker, Karl Schellander and Dawit Tesfaye (2019). Endogenous and Exogenous Modulation of Nrf2 Mediated Oxidative Stress Response in Bovine Granulosa Cells: Potential Implication for Ovarian Function. Int. J. Mol. Sci., 20 (7)

- Sadek, I.I., El-Desokey, W.M.S., **Taqi, M.O.**, Moursy F.S. (2014). Monitoring and description of annual accumulative growing degree days changes in Nile Delta governorates. Life science Journal, 11: 655-665.

- Hassanein, M.K.; Fahim, M.A.; Khalil, A.A.; Abolmaty, S.M.; Refaie, K.M.; **Taqi, M.O.** and Abou Hadid, A.F. (2014). Impact of urbanization on agricultural land losses and climate change Case study: Gharbiah Governorate, Egypt. Journal of American Science, 10: 99-107

- **Taqi, M.O.**; Hassanein, M.K.; Khalil, A.A. (2013). The possible impact of climate change and adaptation options on African livestock: A review. Researcher, 5: 139-145

- Hassanein, M.K.; Abolmaaty, S.M.; Khalil, A.A.; **Taqi, M.O.**; Essa, Yassmin H.; and Shawki, H.H. (2013). Geographical Distribution and Developmental Pattern of Buffalo in Egypt. World Rural Observations, 5: 17-19

- Abdrabbo M.A.; Farag, A.A.; Abul-Soud, M.; Gad El-Mola Manal, M.H.; Moursy Fatma, S.; Sadek, I.I.; Hashem, F.A.; **Taqi, M.O.**; El-Desoky, W.M.S. and Shawki, H.H. (2012). Utilization of satellite imagery for drought monitoring in Egypt. World Rural Observations, 4: 27-37

- Hafez, Y.M., **Taqi, M.O.**, Baiomy, A.A., Medany, M.A. and Abou-Bakr, S. (2011). Physiological and hormonal responses of Egyptian buffalo to different climatic conditions. Egyptian J. Anim. Prod., 48:61-73

b) Articles under preparation

- **Mohamed Omar Taqi**, Mohammed Saeed-Zidane, Samuel Gebremedhn, Dessie Salilew-Wondim, Omar Khdrawy, Ernst Tholen, Christiane Neuhoff, Michael Hoelker, Karl Schellander, Dawit Tesfaye. Differential regulation of transcription factors is associated with bovine granulosa cell functions under oxidative stress condition

- Omar Khadrawy, Samuel Gebremedhn, Dessie Salilew-Wondim, **Mohamed Omar Taqi**, Franca Rings, Christiane Neuhoff, Ernst Tholen, Eva Held, Michael Hoelker, Karl Schellander and Dawit Tesfaye. Quercetin modulates Nrf2-mediated oxidative stress response in bovine preimplantation embryos

c) Abstract / posters

- **M.O. Taqi,** S. Gebremedhn, D. Salilew-Wondim, F. Rings, C. Neuhoff, E. Tholen, E. Held, M. Hoelker, K. Schellander, D. Tesfaye (2019). Cellular and extracellular expression of stress response transcription factors in male and female bovine preimplantation embryos under oxidative stress conditions. IETS conference, New Orleans, Louisiana, USA (Abstr, Poster)

- **Mohamed Omar Taqi,** Samuel Gebremedhn, Mohammed Saeed-Zidane, Dessie Salilew-Wondim, Michael Hölker, Karl Schellander and Dawit Tesfaye (2019). Alterations in expression of transcription factors in bovine granulosa cells exposed to oxidative stress. SSR conference, San José, California, USA (Abstr, Poster)

- **M.O. Taqi,** M. Saeed-Zidane, S. Gebremedhn, D. Salilew-Wondim, F. Rings, E. Held, M. Hölker, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye (2018). Response of male and female bovine embryos to oxidative stress with respect to the expression of developmentally important transcription factors. Vortragstagung der DGfZ und GfT, 12/13.9.2018, Bonn, Germany, (Abstr)

- **M. O. Taqi,** S. Gebremedhn, M. Saeed-Zidane, D. Salilew-Wondim, F. Rings, M. Hölker, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye (2017). Effect of oxidative stress on the expression of transcription factors in bovine granulosa cells. Vortragstagung der DGfZ und GfT, 20/21.9.2017, Stuttgart, Germany, (Abstr)

- **M. O. Taqi**, M. Saeed-Zidane, D. Salilew-Wondim, F. Rings, M. Hölker, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye (2016). Effect of oxidative stress on the expression of transcription factors associated with apoptosis and differentiation in bovine granulosa cells. Vortragstagung der DGfZ und GfT, 20/21.9.2016, Hannover, Germany, (Abstr)

- Hafez, Y.M., **Taqi, M.O.**, Baiomy, A.A., Medany, M.A. and Abou-Bakr, S. (2011). Physiological and hormonal responses of Egyptian buffalo to different climatic conditions. EAAP Conference, Stavanger, Norway (Abstr)

d) Thesis

- M. SC. Thesis: **Mohamed Omar Taqi** (2012): Milk Productivity and Physiological Parameters of Egyptian Buffalo under Climatic Conditions of Giza and Qena Governorates. Faculty of Agriculture, Department of Animal Production, Cairo University, Egypt

e) Scholarships and awards

- German-Egyptian Long-Term Scholarship, GERLS (DAAD) to obtain PhD (2014−2019)

f) Skills

- **Wet lab skills:** Blood samples collection and analysis (haematology and hormone); milk component analysis; cell culture, *in vitro* embryo production technique; molecular genetics, epigenetics (microRNA) and protein (immunoblotting, immunocytochemistry and immunohistochemistry) analysis techniques; exosomes isolation and characterization techniques.
- **Dry lab skills:** Statistical analysis tools (SAS, R and GraphPad Prism); mRNA and Protein analysis software; GIS tools (ArcGIS, ERDAS IMAGINE and GPS software).