

**Epigenetic and exosome-mediated cell-cell communication in  
follicular cells and preimplantation embryos**

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**Mohammed Saeed Zidane**

aus

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Referent :  
Koreferent:  
Tag der mündlichen Prüfung:

Prof. Dr. Karl Schellander  
Prof. Dr. Helga Sauerwein  
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*Dedicated to my parents, lovely sisters, darling wife and  
daughter Hana*



## **Epigenetic and exosome-mediated cell-cell communication in follicular cells and preimplantation embryos**

Follicular cells and preimplantation embryos communication with their in vivo or in vitro environments via mechanisms including cell adhesion and extracellular vesicles is crucially essential for normal oocyte and embryo development and subsequently successful pregnancy. Therefore, this dissertation aims to assess the potential role of exosomes in granulosa cells' oxidative stress response and figure out the epigenetic role of DNA methylation in the regulation of focal adhesion pathway in preimplantation embryos. For that, two main experiments were conducted; where bovine granulosa cells were cultured under oxidative stress conditions induced by H<sub>2</sub>O<sub>2</sub>. Exosomes were isolated from culture media and subjected to mRNA quantification analysis. Thereafter, control and H<sub>2</sub>O<sub>2</sub>-stressed cells were co-cultured with non-stress or stress-released exosomes. Cells of all experimental groups were subjected to ROS level, mitochondrial activity, cell cycle and cell proliferation assays as well as mRNA and protein expression analyses. In parallel, bovine embryos were cultured in media supplemented with EGF and/or HA. Blastocysts were subjected to ROS level, TUNEL, cryopreservation assays and protein analysis. Moreover, embryos were cultured in media supplemented with epidermal growth factor (EGF) and hyaluronic acid (HA) pre, post and during embryonic genome activation (EGA). Blastocysts from all groups were subjected to mRNA expression and DNA methylation analysis of genes involved in focal adhesion pathway. Results revealed that, H<sub>2</sub>O<sub>2</sub>-induced oxidative stress significantly increased ROS level as well as mRNA and protein expression of Nrf2 and antioxidant genes. Stress-released exosomes were enriched with mRNA of Nrf2 and selective antioxidants (CAT, and TXN1). Granulosa cells co-cultured with stress-released exosomes exhibited higher mRNA and protein levels of Nrf2 and its downstream antioxidants which in turn reduced ROS level and enhanced mitochondrial activity and cell proliferation under oxidative stress conditions. Furthermore, culture media supplemented with EGF and/or HA altered the DNA methylation pattern of genes involved in focal adhesion pathway associated with significant high mRNA and protein levels. The supplementation of EGF and HA particularly during EGA had a positive effect on mRNA expression of focal adhesion genes. Blastocysts with higher focal adhesion mRNA and protein levels had low ROS level and apoptosis with high cryotolerance. These findings highlighted the role of exosomes and DNA methylation in cell-cell communications and subsequently cell function and embryo quality under suboptimal conditions.

## **Epigenetische und exosomal-vermittelte Kommunikation von Follikelzellen und präimplantierten Embryonen**

Die Kommunikation von Follikelzellen und präimplantativen Embryonen mit ihren *in vivo* oder *in vitro* Umgebungen erfolgt über Mechanismen wie z. B. Zelladhäsion und extrazelluläre Vesikel. Diese sind ausschlaggebend für eine normale Entwicklung der Eizelle, des Embryos sowie eine anschließende erfolgreiche Trächtigkeit. Daher ist das Ziel der vorliegende Dissertation, die Bedeutung von Exosomen in der oxidativen Stressantwort von Granulosazellen zu untersuchen. Zusätzlich soll die epigenetische Funktion der DNA-Methylierung auf die Regulierung der fokalen Adhäsion in der Präimplantation von Embryonen betrachtet werden. Dazu wurden zwei Experimente durchgeführt, in welchen bovine Granulosazellen unter oxidativen Stressbedingungen mittels  $H_2O_2$  kultiviert wurden. Die Exosomen wurden aus den Kulturmedien isoliert und deren mRNA quantifiziert. Anschließend wurden die Zellen der Kontroll- sowie der  $H_2O_2$ -behandelten Gruppe mit Exosomen co-kultiviert, die entweder unter der  $H_2O_2$ -Behandlung oder ohne diese gebildet wurden. Aus allen experimentellen Gruppen wurden Zellen hinsichtlich ihres ROS-Niveaus, ihrer mitochondrialen Aktivität, ihres Zellzyklus und der Zellproliferation untersucht sowie die Expression von mRNA und Proteinen bestimmt. Zeitgleich wurden bovine Embryonen in Medien kultiviert, die mit EGF und/oder HA angereichert wurden. Blastozysten wurden hinsichtlich des ROS-Niveaus, TUNEL, der Kryokonservierung und der Proteinexpression analysiert. Darüber hinaus wurden Embryonen vor, nach und während der Embryonale Genomaktivierung (EGA) in einem Medium kultiviert das mit Epidermaler Wachstums Faktor (EGF) und Hyaluronsäure (HA) angereichert wurde. Blastozysten aller Untersuchungsgruppen wurden hinsichtlich ihrer mRNA-Expression und DNA-Methylierung von Genen, die in den Verlauf der Fokalahäsion involviert sind, analysiert. Die Ergebnisse zeigten, dass der durch  $H_2O_2$  induzierte oxidative Stress die ROS-Niveaus, die mRNA- und Protein-Expression von Nrf2 und antioxidativen Genen signifikant gesteigert hat. Unter Belastung ausgeschüttete Exosomen waren mit mRNA von Nrf2 und selektiven Antioxidantien (CAT und TXN1) angereichert. Granulosazellen, die mit unter Stress freigesetzten Exosomen co-kultiviert wurden, wiesen höhere mRNA und Proteinkonzentrationen von Nrf2 sowie dessen nachfolgenden Antioxidantien auf. Diese reduzierten wiederum das ROS-Niveau, verstärkten die mitochondriale Aktivität und Zellproliferation unter oxidativem Stress. Weiterhin veränderte die mit EGF und/oder HA angereicherten Medien das Muster der DNA-Methylierung von Genen, die in den Verlauf der fokalen Adhäsion involviert sind und führte zu einer signifikant erhöhten mRNA- und Proteinexpression dieser Gene. Vor allem die Ergänzung von EGF und HA während der EGA zeigte einen positiven Effekt auf die mRNA Expression der Gene der fokalen Adhäsion. Blastozysten mit erhöhten mRNA und Proteinlevels von Genen der fokalen Adhäsion zeigten niedrigere ROS-Niveaus und Zelltod bei hoher Kryotoleranz. Diese Ergebnisse betonen die Rolle von Exosomen und der DNA-Methylierung in der Kommunikation zwischen Zellen, der anschließenden Zellfunktion und der Embryoqualität unter suboptimalen Bedingungen.

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## List of abbreviations

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ACTB	$\beta$ -actin
ACTG1	Actin gamma 1
Alix	ALG-2 interacting protein X
BCL2L1	BCL2 like 1
BM	Basic media
BSA	Bovine serum albumin
BSA-FAF	Bovine serum albumin fatty acids free
CASP3	Caspase 3, poptosis-related cysteine peptidase
CAT	Catalase
CCDN2	Enzyme-linked immunosorbent assay
CD44	CD44 molecule
CD63	CD63 molecule
cDNA	Complementray DNA
CO <sub>2</sub>	Carbondioxide
COL1A2	Collagen, type I, alpha
COL4A1	Collagen, type IV, alpha 1
COCs	Cumulus oocyte complexes
Ct	Threshold cycle
CYCS	Cytochrome c
DAPI	4',6-Diamidin-2'-phenylindoldihydrochlorid
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
dNTPs	Deoxyribonucleoside triphosphate
D-PBS	Dulbecco's phosphate-buffered saline
E. coli	Escherichia coli
ECM	Extracellular matrix
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
FA	Focal adhesion
FAK	Focal adhesion kinase
FITC	Fluoresceinisothiocyanat
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HA	Hyaluronic acid
HMMR	Hyaluronan-mediated motility receptor
HMOX1	Heme oxygenase 1
ICM	Inner cell mass
IVF	In vitro fertilization
IVP	In vitro production
kDa	Kilo Dalton
Keap1	Kelch like ECH associated protein 1
MAPK	Mitogen-activated protein kinases
miRNA	Micro RNAs
PI	Propidium iodide
mRNA	Messenger RNA
ncRNA	Noncoding RNA
NormalExo	Exosomes released under normal conditions
NQO1	NAD(P)H quinone dehydrogenase 1
Nrf2	Nuclear factor, erythroid 2 like 2
OD	Optical density
PAK4	p21 protein (Cdc42/Rac)-activated kinase 4
PBS	Phosphate-buffered saline
PBS-CMF	Ca <sup>2+</sup> /Mg <sup>2+</sup> free 1x phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PRDX1	Peroxiredoxin 1
PTEN	Phosphatase and tensin homolog
PVA	Poly vinyl acetate
qRT-PCR	Quantitative real-time polymerase chain reaction

## XII

RAC1	Ras-related C3 botulinum toxin substrate 1
ROS	Reactive oxygen species
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
SEM	Standard error
SOD1	Superoxide dismutase 1
SOF	Synthetic oviductal fluid
STAR1	Steroidogenic acute regulatory protein
StressExo	Exosomes released under oxidative stress conditions
TE	Trophoectoderm
TUNEL	Terminal deoxynucleotil transferase uracil nick end labeling
TXN1	Thioredoxin
V/V	Volume per volume
VCL	Vinculin
18S	18S ribosomal RNA

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Chapter 1: General overview



## 1.1 Introduction

Investment of the assisted reproductive technologies and molecular biology power to minimize the impacts of environmental and exogenous factors on animal reproductive traits through the study of genetic pathways and epigenetic mechanisms would help to clarify and understand critical points regarding to the reproductive performance variation between animals within breed. The application of in vitro embryo production is one of the reproductive technologies that facilitated the understanding of many molecular mechanisms of how/why having specific phenotypes (Nandi et al. 2006). Oxidative stress and suboptimal culture conditions are strongly considerable in vivo and in vitro factors in reproductive and molecular biology studies (Gad et al. 2012, Amin et al. 2014). The cells/tissues respond and react with the extracellular signals and the changeable surrounding environment via communicating with other cells and extracellular matrix through several mechanisms including cell adhesion and secretion of extracellular vesicles (Corrado et al. 2013). Hence, here we studied the effect of suboptimal culture condition factors such as oxidative stress and in vitro culture media on the regulation of cell response and communication via exosomes and DNA methylation in granulosa cells and preimplantation embryos. Exosomes are a member of extracellular vesicles with ranged size of 30-150 nm, they are found to be secreted in all types of cells and fluids that have been studied so far (Théry et al. 2002). Exosomes carry different genetic information molecules (DNA, mRNA, ncRNA, proteins, lipids etc.) depends on from where and how they secreted (Santonocito et al. 2014). Exosomal cargo molecules can be transferred to neighbor or long distance cell/tissue resulting in signaling responses (Di Pietro 2016). On the other hand, focal adhesion is one of fundamental cell communication pathways and it refers to cellular mechanical link between actin cytoskeleton and extracellular matrix mediated by integrin receptors which work in a dual fashion in-out and out- in cell (Saif et al. 2003). Focal adhesion involves in several physiological functions including proliferation, differentiation, motility and survival (Geiger et al. 2001, Dumbauld et al. 2013). Furthermore, it is sensitive to various environmental factors (Khalili and Ahmad 2015) and the changes in the surrounding environment. Previous results indicated that alternative culture conditions altered the mRNA expression and DNA methylation patterns of focal adhesion pathway related genes (Gad et al. 2012, Salilew-Wondim et al. 2015). DNA methylation is an epigenetic regulatory mechanism refers to the addition of methyl

group to cytosine nucleotide in the position of CpG which resulting in alteration of gene expression (Robertson 2005, Jin et al. 2011). An overview regarding to animal fertility, stress factors, cell communication, exosomes, focal adhesion and epigenetic terms is highlighted in the following points.

### 1.1.2 Animal fertility and environmental stress

Fertility is a wide term which is influenced by a network of genetic, environmental, nutritional, hormonal, physiopathological and management factors. Due to the low heritability, variations in the fertility traits are mostly determined by non-genetic or environmental factors (Dash et al. 2016). Stress has been defined as the reaction of the body to stimuli that disturb the normal physiological homeostasis, often with the negative and harmful effects on production and reproduction (Khansari et al. 1990). Also, it is known as the failure to cope up with the environment, a phenomenon that shows the inability of an animal to achieve its genetic potential (Dobson and Smith 2000). There are more than one environmental stress which affecting the performance of the animal, like, heat stress (Li et al. 2016), pH (Dagilgan et al. 2015), malnutrition (van Hoeck et al. 2011), osmotic stress (Agca et al. 2000) and etc., and subsequently oxidative stress (Agca et al. 2000, Roth 2015). Combination of two stressors has a greater influence on animal growth and reproduction. For instance, exposing the animals to nutritional or heat stress as separated stressors has no severe effect on growth and reproduction compared with exposing to both stressors simultaneously (Sejian et al. 2011).

Heat stress alone or a combined with other stress factors is deemed as one of the main factors affecting the dairy cattle productive and reproductive performance. Lactating cows are found to be more sensitive to heat stress as a result of high metabolic rate which is associated with high milk production. Moreover, the heat stress may compromise the reproductive efficiency of both sexes. Here, we will summarize the potential impact of heat stress on female reproduction. There are various mechanisms by which heat stress can negatively affect growth and development of the oocytes and subsequently embryo development and quality (Wolfenson et al. 2000).

The foremost mechanism is the changing of blood hormonal profile by activating hypothalamic-pituitary-adrenal (HPA) axis, which subsequently excites the pituitary gland to release adrenocorticotrophic hormone (ATCH) as shown in Figure 1.1 (Krishnan

et al. 2017). The chronic release of ACTH inhibits the follicular development and ovulation via altering the follicular selection efficiency and dominance (Al-Katanani et al. 2002). Additionally, the high level of glucocorticoids during heat stress is responsible for the inhibition of oocyte maturation. Also, corticotrophic releasing hormone (CRH) inhibits the ovarian steroidogenesis, leading to reduce length and intensity of estrus expression (Masoumi and Derensis 2013). The decline in estradiol secretion during heat stress is attributed to reduction of granulosa cells aromatase activity and viability (Krishnan et al. 2017).

Subsequently, the concentration of progesterone (P4) in plasma, in dominant follicle, is reduced, resulting in the small size of ovulatory follicles with low tonic LH stimulation of luteinization and steroidogenesis (Wakayo et al. 2015). Consequently, the low progesterone level compromises the endometrial function and the subsequent embryo development. Furthermore, increasing plasma concentration of P4 in summer season may cause infertility (Roy and Prakash 2007).

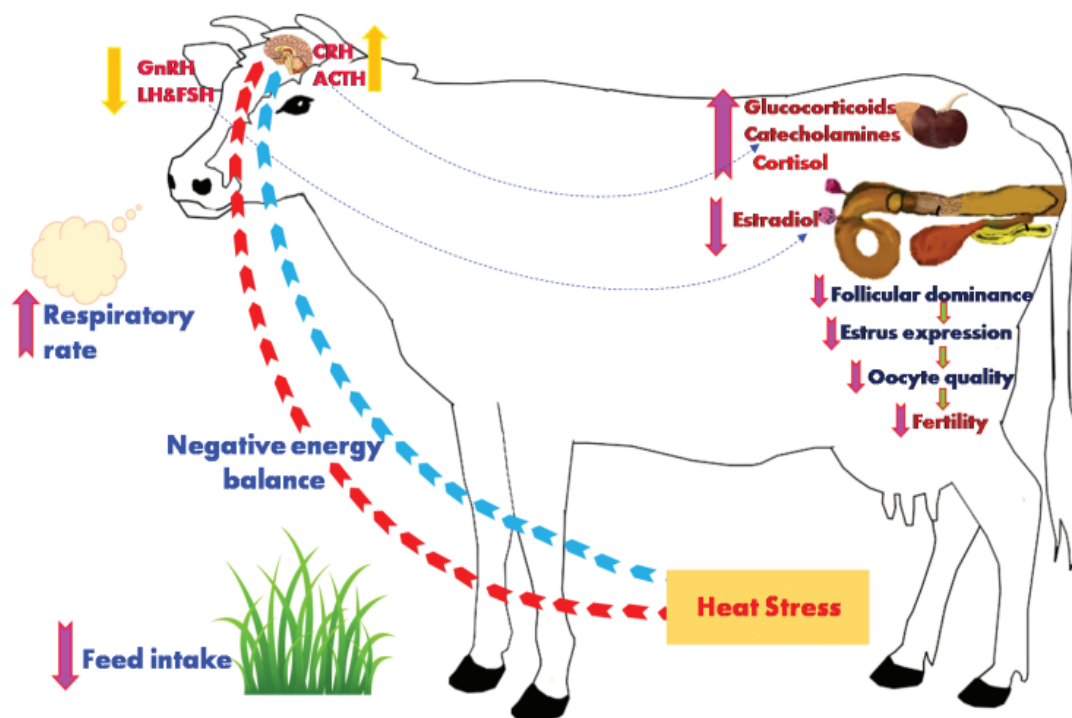


Figure 1.1. Impact of heat stress on dairy cattle reproductive performance mediated by changing of blood hormonal profile (Krishnan et al. 2017)

The heat stress mediated disturbance of steroid hormones such as luteinizing hormone and follicle-stimulating hormone leads to ovarian dysfunction, reduction of oocyte competency, impaired fertilization success and embryo development (Naqvi et al.

2012). Furthermore, incompetent oocytes lose the transcriptional ability to produce heat shock protein 70 (HSP70) in response to heat shock (Ahmed et al. 2015) which also negatively affect the formation of transcripts required for embryo development (Edwards and Hansen 1997). On the other hand, the impairment of protein synthesis and elevation of free radicals in the oocytes as a result of stress compromise the zona pellucida layer and the oocyte cytoplasm which in turn impair sperm penetration (Wolfenson et al. 2000). Interestingly, the mitochondrial activity is reduced in the germinal vesicle stage oocytes and their surrounding cumulus cells which exposed to heat shock (Paula-Lopes et al. 2012). Moreover, the alteration in the mitochondrial activity is associated with activation of apoptosis pathways (Roth 2015). Regarding to that, exposing oocytes to stress factors during maturation activates the caspase 2, 3, and 7, which are related to apoptosis cascades (Roth and Hansen 2004).

The embryo mortality is deemed to be another factor affected by heat stress. During the first two weeks of pregnancy, increasing sensitivity to heat stress was noticed in bovine embryos (Ahmed et al. 2015, Wakayo et al. 2015). Embryo death induced by heat stress is attributed to oxidative cell damage, reduction in pregnancy recognition and activation of genes related to apoptosis (Krishnan et al. 2017). On the other hand, in terms of in vitro embryo production it is found that the inadequate feeding as a result of heat stress has a negative effect on oocyte quality, which results in lower cleavage and blastocyst formation. Meanwhile, the overfeeding increased serum glucose and insulin in ewes (Grazul-Bilska et al. 2012) and cattle (Sales et al. 2015). Moreover, High blood glucose level was associated with developmental block during embryonic genome activation (EGA), which induced delaying of preimplantation development and subsequently low embryo quality (Furnus et al. 1997).

### 1.1.3 Oxidative stress and fertility

Oxidative stress is caused by a disturbance in the balance between formation of free radicals including reactive oxygen species (ROS) and their scavengers by enzymatic and non-enzymatic antioxidant molecules. This imbalance may be caused by internal sources such as mitochondrial dysfunction or external sources such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^-$ ), which are known as free radicals (Martindale and Holbrook 2002). Furthermore, ROS may be produced in the cell as a byproduct of normal aerobic metabolism, or as second messenger in various signal transduction

pathways (Martindale and Holbrook 2002). Approximately 1–3% of the O<sub>2</sub> consumed by cells is metabolized into ROS in the mitochondria (Wu et al., 2014), which can interact with and damage other molecules, including DNA, protein, and lipid (Martindale and Holbrook 2002). Furthermore, it does not only cause direct DNA damage, but also affects the DNA repair mechanisms along with alterations in important check points in cell cycle (Barzilai and Yamamoto, 2004). Moreover, it may influence cell structure such as cell membrane, which can compromise the mitochondrial integrity and activity (Kadenbach et al., 2004). Martindale and Holbrook (2002) mentioned that the cell response to ROS is ranging from proliferation to growth arrest or cell death depending on the level of ROS accumulation.

ROS is working as double-edged sword. It is already known that the normal physiological range of ROS is involved in several cell functions such as cell proliferation, differentiation and migration (Agarwal et al. 2004, Rhee 2006). For instance, ROS is produced during metabolism in the ovary (Prasad et al. 2016) and is involved in various processes in female reproductive tract like folliculogenesis, follicle maturation, ovulation and corpus luteal function (Agarwal et al. 2005). Interestingly, a moderate level of ROS inside the follicle microenvironment is beneficial for diplotene-arrested oocytes to resume meiosis (Chaube et al. 2006, Prasad et al. 2016, Tiwari et al. 2017). Meanwhile, the high physiological level of ROS may be associated with final oocyte maturation (Prasad et al. 2016).

However, supra-physiological range of ROS generation as a result of stress, is related to cell cycle arrest and apoptosis in oocyte (Chaube et al. 2005, Tripathi et al. 2009). Furthermore, oxidative stress provokes disorders of chromosomal segregation and oocyte fragmentation resulting in failure of fertilization and infertility (Tatone et al. 2015, Prasad et al. 2016). Likewise, the oxidative stress induced apoptosis in granulosa cells, which are surrounding the oocyte, disturbs the internal communication between oocyte and granulosa cells and subsequently the nutrient supply that in turn negatively impact the oocyte quality (Chaube et al. 2014). Furthermore, embryo cultured under oxidative stress resulted in reduced number of embryos that reached to blastocyst stage (Amin et al. 2014). However, the sensitivity of embryo to oxidative stress also differed according to developmental stage and embryonic genome activation (EGA) (Gad et al. 2012). On the other hand, the sperm concentration is a critical factor for inducing oxidative stress which leading to decrease cell viability, but had no effect on

mitochondrial activity of sperm (Murphy et al. 2013). It also affects the sperm DNA integrity which consequently reduces cleavage rate and embryo quality (Simoes et al. 2013). Indeed, the balance between pro- and anti-oxidants is important for the normal physiological function of the cell. cell to protect against oxidative stress and to decrease levels of ROS which could be led to survival or death the cell such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway (Amin et al. 2014) and mitogen-activated protein kinases (MAPK) pathway (Martindale and Holbrook 2002). Accordingly, manipulating of the oxidation status is recommended as a potential strategy to conquer the adversely impacts of heat stress, which in turn leading to oxidative stress, on fertility. On the other hand, there are some compounds which play as an antioxidant including enzymatic as well as non-enzymatic nature. It is now common to supplement antioxidant compounds to culture media because insufficient of embryo antioxidant in order to preventing from oxidative stress (Ali et al. 2003), such as vitamins A, (Livingston et al. 2004) C, and E (Olson et al. 1990; Paula-Lopes et al. 2003), could be used each of them alone or combined, cysteine (Ali et al., 2003); Glutathione and Hypotaurine (Donnelly et al. 2000), as non-enzymatic compound. In addition to enzymatic compounds that include catalase (CAT) (Chi et al. 2008), superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Gupta et al., 2009). Additionally, several defense mechanisms are present in cell to protect against oxidative stress and to decrease levels of ROS which could be led to survival or death the cell such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway (Amin et al. 2014) and Mitogen-Activated Protein Kinases (MAPK) pathway (Martindale and Holbrook 2002). Accordingly, manipulating of the oxidation status is recommended as a potential strategy to conquer the adversely impacts of heat stress, which in turn leading to oxidative stress, on fertility.

#### 1.1.4 In vitro embryo production and suboptimal culture conditions

In vitro embryo production is among bovine reproductive biotechnologies which are currently believed to be important in breeding and husbandry aspects (Kocyigit 2016). The production of in vitro embryos has increased year by year in cattle (Blondin 2015); however the percentage of blastocyst rate remains nearly 40% (Kepkova et al. 2011; Pellegrino et al. 2016). On the other hand, numerous studies stated the differences of bovine in vitro embryos compared with in vivo counterparts in terms of embryonic

morphology (Greve et al. 1995), development (van Soom et al. 1997), metabolism (Khurana and Niemann 2000), and gene expression (Niemann and Wrenzycki 2000) which result in low quality of in vitro produced embryos (Lonergan et al. 2003). In vitro culture conditions (Figure 1.2) including culture media, oxygen level, pH, temperature and etc. during in vitro oocyte maturation, fertilization and embryo culture were found to alter the embryonic genetic pathways and epigenetic mechanisms which in turn resulted in reduction of embryo development and quality (Urrego et al. 2014). Despite of all attempts that take place so far to improve the in vitro culture conditions, the in vitro production of bovine embryos is still suboptimal (Do et al. 2016). Moreover, in vivo and in vitro alternative culture conditions reform the expression pattern of several molecular pathways involved in embryo development (Gad et al. 2012).

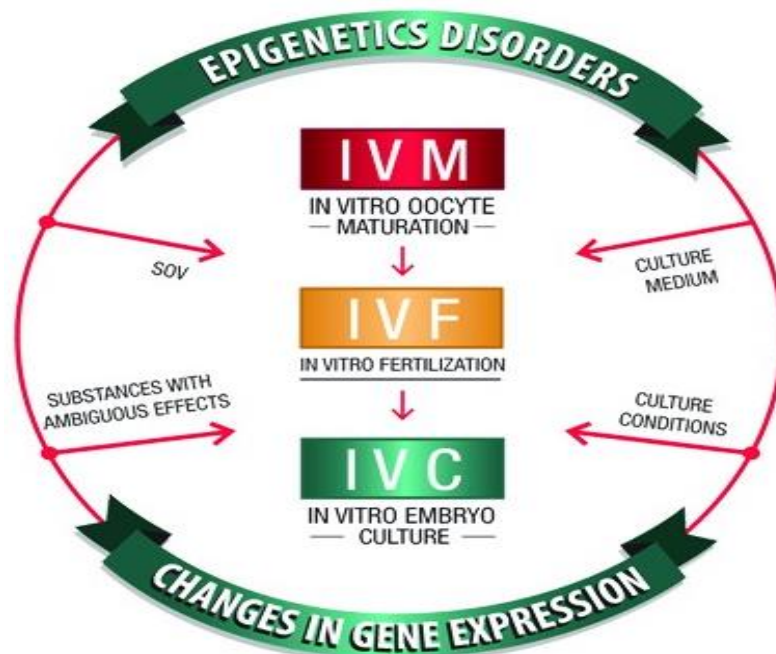


Figure 1.2. Suboptimal culture conditions altering expression pattern and epigenetic disorders during oocyte in vitro embryo production maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) in vitro embryo production (Urrego et al. 2014)

#### 1.1.5 Exosomes and focal adhesion mediated cell-to-cell communication

Cell-to-cell communication is the backbone mechanism that a multicellular organism relies on to maintain homeostasis and normal cellular functions (Lee et al. 2014). Cells communicate with their surrounding environment through several pathways including direct surface-surface communication mediated by membrane-bound proteins and lipids or via secretion of growth factors, cytokines, hormones, chemokines and extracellular

vesicles such as microvesicles and exosomes (Corrado et al. 2013). As shown at figure 1.3 exosomes are nano-sized extracellular membrane vesicles ranged from 30 to 150 nm in size (Li et al. 2014, Sharma et al. 2016) and contain a cargo of DNA, mRNAs, microRNAs, proteins, lipids etc. and have a pivotal role in cell-to-cell communication (Muralidharan-Chari et al. 2010). Exosomes are released through exocytosis process to the extracellular space nearly in all types of cells including preimplantation embryos and found in various biological fluids (Pan et al. 1985, They et al. 2002, Keller et al. 2006, Sluijter et al. 2014, Frydrychowicz et al. 2015). Regardless of origin, exosomes have similar and specific surface proteins such as CD9, CD63, CD81 and Alix (Vlassov et al. 2012).

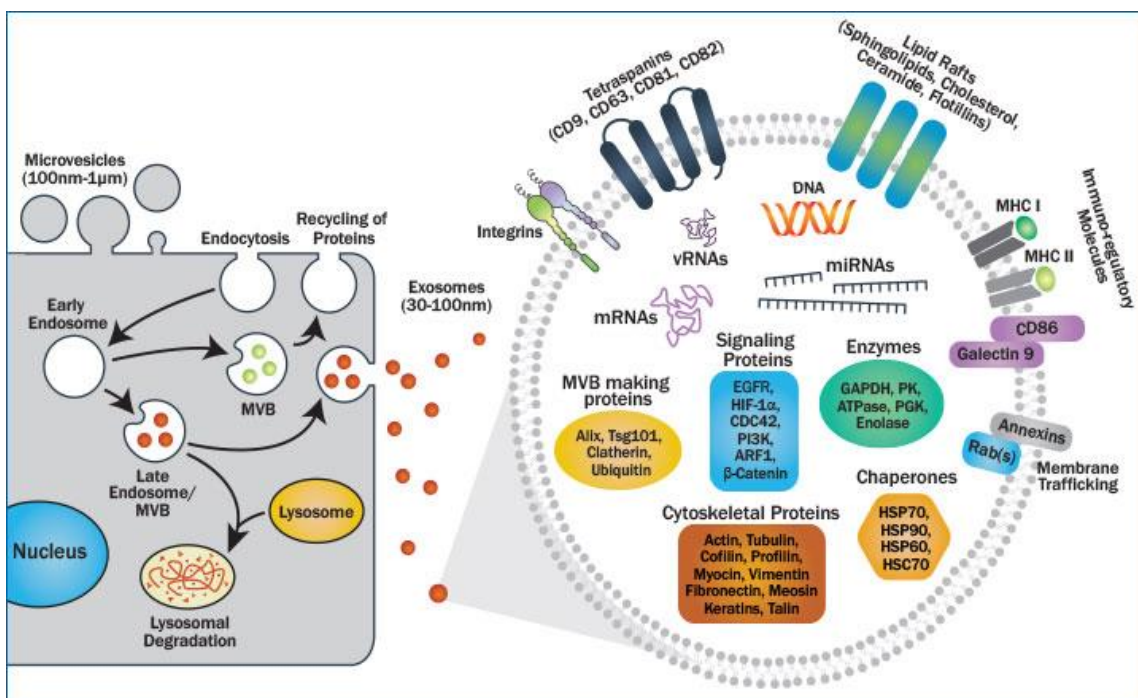


Figure 1.3. Exosomes secretion, composition and their cargo of genetic information (<https://www.novusbio.com>)

Interestingly, exosomes have been isolated from follicular fluids in mare (da Silveira et al. 2012), woman (Santonocito et al. 2014) and cow (Sohel et al. 2013; Navakanitworakul et al. 2016), which indicate their importance within the follicular compartment. They were found to have a role in supporting cumulus cells expansion and to alter gene expression of cumulus-oocyte-complex (COC) as well (Hung et al. 2015). Moreover, exosomes are differentiated in their quality and quantity under different environment conditions (Ailawadi et al. 2015; Navakanitworakul et al. 2016, Harmati et al. 2017) and highly regulated by various stresses such as diseases, heat



stress including oxidative stress (Ailawadi et al. 2015). Indeed, exosomes released under stress conditions obviously differed in their RNA and protein contents compared to exosomes released under normal conditions (Eldh et al. 2010). Several studies demonstrated the vital role of exosomes in horizontal genetic transfer during preimplantation embryo development (Qu et al. 2017).

In addition, cell adhesion to extracellular matrix (ECM) is a fundamental process involved in cell interaction and communication with surrounding environment. Cell-ECM adhesion is required for normal cellular functions during organogenesis and embryonic development (Wu 2007). One of the identical structure of cell-ECM adhesion is focal adhesion, it refers to ECM mechanical link with intercellular actin bundles where integrins are mediating dual fashion signals (Wu 2007). This communication between the ECM and the cytoskeleton mediated by focal adhesion provides vascular wall components to respond to biochemical and biophysical signals from cellular surrounding environment. This “outside-in” signaling mediated by integrins alters cytoskeletal organization and gene expression resulted in specific cellular response and function (Figure 1.4A). On the other hand, focal adhesion also provides an “inside-out” integrins-mediated cellular signaling component (Figure 1.4B) which results in conformational change the ECM and the behavior of neighboring cells (Romer et al. 2006; Millard et al. 2011). Focal adhesion has a vital role in several biological processes such as cell motility, differentiation, migration, proliferation and cell survival (Kaneko et al. 2012, Wrighton 2013). Four major factors including (1) the biophysical and biochemical properties of the ECM, (2) integrin activation, (3) the contraction state of the cytoskeleton and (4) the specific cellular and tissue milieu in which these events occur are influencing the assembly rate, size, specific constituency, signaling repertoire and functional impact of focal adhesion (Romer et al. 2006).

Although the forward and reverse genetics provide strong approaches to determine the fundamental roles of focal adhesion proteins in embryo development, however, little is known about focal adhesion regulation during embryo development (Wu 2007). Many of focal adhesion proteins, including  $\beta 1$  integrin,  $\alpha 4$  integrin,  $\alpha 5$  integrin, talin, paxillin, vinculin, focal adhesion kinase and integrin like kinase are essential for vertebrates' embryonic development, and functional loss of these proteins during embryogenesis would affect cell-ECM adhesion, cytoskeletal organization, polarity, migration and/or survival of embryos (Bladt et al. 2003).

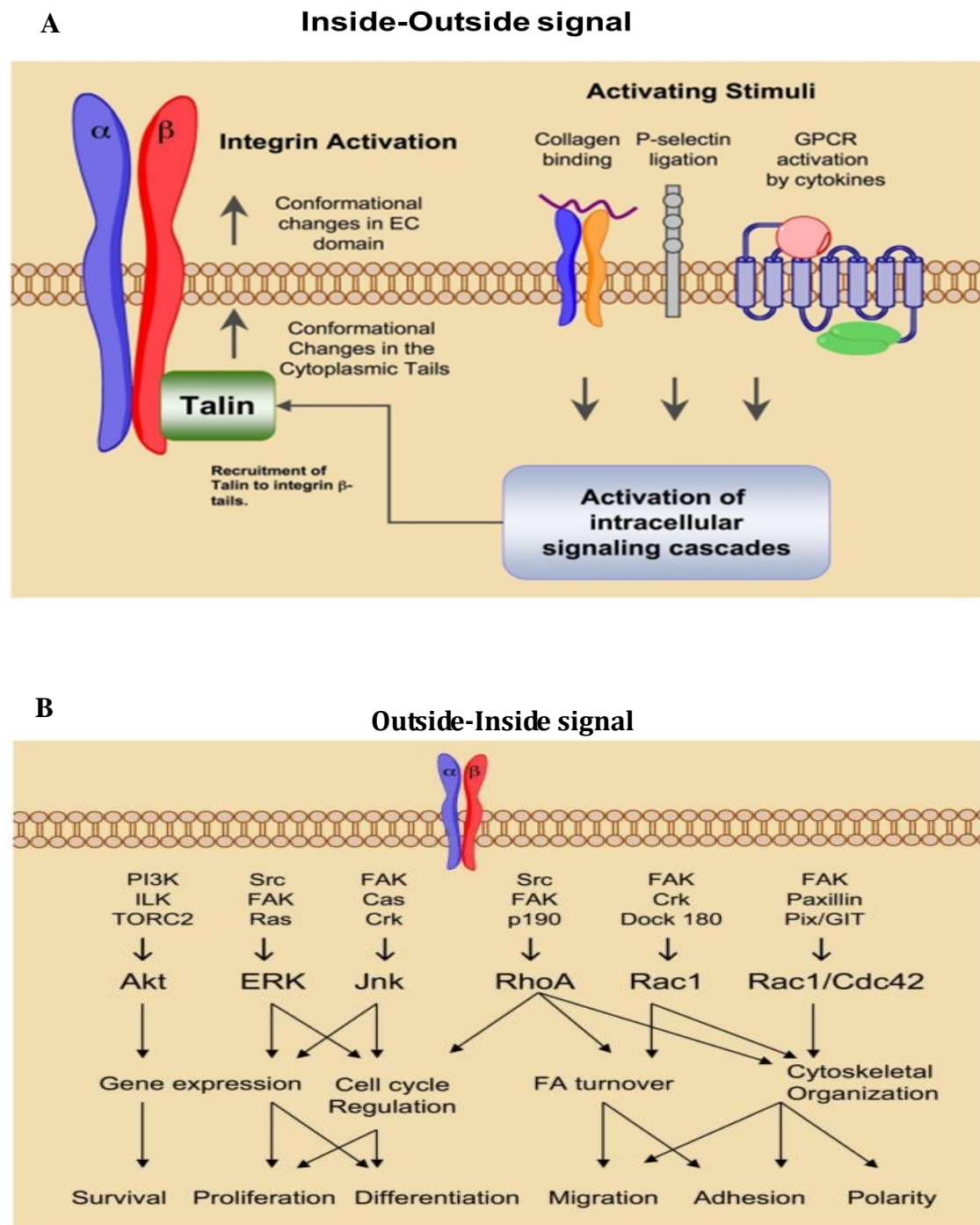


Figure 1.4. Focal adhesion pathway cellular signals communication with ECM. Inside-outside signal (A) where focal adhesion proteins bind to integrin receptors resulting in conformational changes in extracellular (EC) domain and activating stimuli while, (B) represents outside-inside signal where integrins bind to extracellular component which affect the recruitment of focal adhesion proteins and cellular cytoskeleton leading to cellular functions (Millard et al. 2011)

## 1.1.6 Epigenetic regulatory mechanisms and DNA methylation

Epigenetics is the study of mechanisms that alter gene expression and subsequently phenotype without changing in the underlying gene sequence (Goldberg et al. 2007, Handy et al. 2011). These epigenetic regulatory mechanisms include chromatin remodeling, histone modifications, DNA methylation and non-coding RNAs such as microRNAs regulate the gene expression on transcription or post transcription levels as shown in figure 1.5 (Puumala and Hoyme 2015).

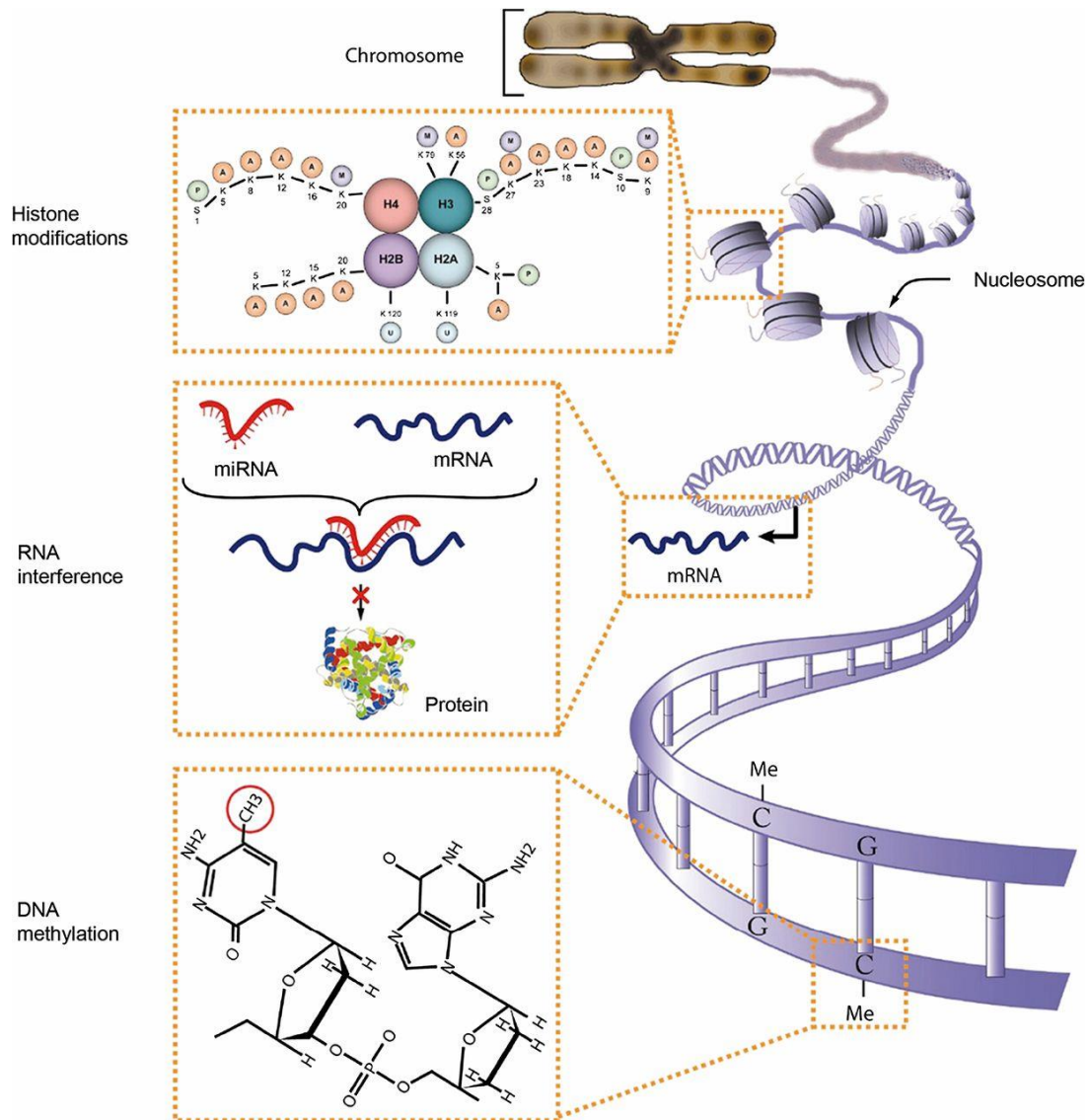


Figure 1.5. Epigenetic regulatory mechanisms including histone modifications, microRNAs (miRNA) and DNA methylation which involved in gene expression regulation (Kim et al. 2011)

There are several environmental and physiological factors such as climate, nutrition, physiological status, sex, reproductive technologies, diseases and aging that are found to modify epigenetic regulatory mechanisms (Ibeagha-Awemu and Zhao 2015). These epigenetic modifications are influencing livestock (cattle, sheep, goat, and pig) production and reproduction (Singh et al. 2010, Urrego et al. 2014). Moreover, the modifications of epigenetic mechanisms can be transferred during cell division or between generations of a species (Ling and Groop 2009). Reproductive and productive efficiency of farm animals has improved with the application of new reproductive technologies such as in vitro embryo production (Pontes et al. 2010). However, the low quality of in vitro produced preimplantation embryos associated with more loss during pregnancy compared to in vivo counterparts (Lonergan et al. 2003), resulted from transcription profile differences between in vitro and in vivo embryos (Tesfaye et al. 2003, Clemente et al. 2011, Aksu et al. 2012, Betsha et al. 2013, Dos Santos-Neto et al. 2017) associated with epigenetic dysregulation which take place throughout in vitro production processes (Urrego et al. 2014).

DNA methylation is a well-known epigenetic mechanism that regulates gene expression (Bird 1986) and in general but not always is associated with repression of genes (Bird 1986). DNA methylation refers to methylation of the cytosine nucleotide by the addition of methyl group (CH<sub>3</sub>) to C5 of the cytosine residue in the position of CpG dinucleotide (Bird 1986, Jin et al. 2011) via DNA methyltransferases enzymes including DNMT1 which is responsible for the maintenance of DNA methylation during DNA replication while, DNMT3A, DNMT3B that are responsible for de-novo DNA methylation during development (Young and Beaujean 2004, Jin et al. 2011). However, there is new evidence indicated that, all of DNA methylation enzymes are involved in DNA maintenance and de-novo methylation during replication and development (Riggs and Xiong 2004, Egger et al. 2006). Moreover, DNA methyltransferases enzymes are required for normal embryonic development (Yin et al. 2012), deletion of one of DNA methyltransferase enzymes resulted in embryonic death (Li et al. 1992, Okano et al. 1999, Li 2002).

DNA methylation has a unique profile for a particular tissue/cell, which maintained after DNA replication and cell differentiation for cell specific gene expression although an organism tissues and cells contain the same genomic information (Ibeagha-Awemu and Zhao 2015). During preimplantation mammalian embryo development the DNA

methylation is dynamically changed (Young and Beaujean 2004) to regulate embryo development in a sex and stage specific manner (Dobbs et al. 2013). In cow, following fertilization until EGA stage (8-16 cell stage) the embryo remains in a state of transcriptional quiescence which is associated with decline in global DNA methylation pattern (Morgan et al. 2004); moreover, the reprogramming of global DNA methylation is required for EGA and development (Dobbs et al. 2013). The DNA methylation starts to increase in gender and cell type specific manner; male bovine embryos are higher in global DNA methylation profile than female embryos at the blastocyst stage while trophectoderm cells are higher than inner cell mass in DNA methylation pattern (Dobbs et al. 2013). Moreover, in vitro and in vivo produced embryos differ in their global DNA methylation profile (Wrenzycki and Niemann 2003), DNA methylation is sensitive to environmental and nutritional influences and the DNA methylation changes lead to alteration of transcriptomic profiles and subsequently influence productivity (Choi and Friso 2010, Jang and Serra 2014). Environment conditions including suboptimal in vitro culture conditions result in DNA methylation changes during embryo development (Katari et al. 2009, Reis e Silva et al. 2012).

Our previous study revealed that alternative culture environment resulted in global DNA methylation changes in bovine preimplantation embryos that showed higher hyper and hypo methylated probes in in vitro produced embryos compared with in vivo counterparts (Salilew-Wondim et al. 2015). The dynamic changes in DNA methylation pattern of embryos during in vitro development may rely on the epigenetic adaptability of embryos resulting from the cellular interactions with extracellular environment via cell adhesion to ECM molecules. Focal adhesion pathway showed differential DNA methylation pattern at different developmental stages of bovine preimplantation embryos exposed to alternative in vivo or in vitro culture conditions (Salilew-Wondim et al. un-published data). So far, few is known about the epigenetic regulation of focal adhesion pathway in bovine embryos produced under different developmental conditions. DNA methylation could be used as remark for embryo quality. Taken together we aimed to figure out the association between embryonic development and quality and regulation of focal adhesion pathway.

## 1.2 Hypothesis and objectives

Here, we hypothesize that oxidative stress conditions alter cell communication mechanisms via induction of exosomes that carry specific antioxidant molecules (chapter 2) and DNA methylation mediate the regulation of focal adhesion pathway of preimplantation embryos produced under different culture conditions (chapter 3). Therefore, objectives were to investigate:

1. Potential role of exosomes in molecular defense mechanism of bovine granulosa cells exposed to oxidative stress conditions (chapter 2).
2. The role of DNA methylation in the regulation of focal adhesion pathway and subsequently development and quality of bovine in vitro produced embryos supplemented with epidermal growth factor and hyaluronic acid (chapter 3).

### 1.3 Materials and methods

In order to achieve our goals, several materials and methods were performed as detailed described in the respective chapters of this dissertation. However, the main technical methods used during the study are highlighted here.

#### 1.3.1 Experimental setup and ethics approval

Two independent experiments were designed to investigate our objectives. The first experiment was performed on bovine granulosa cells and exosomes released under in vitro oxidative stress conditions induced by H<sub>2</sub>O<sub>2</sub>. For this, bovine ovaries were collected from a local slaughterhouse and granulosa cells were aspirated from small follicles (3-5 mm diameter). Collected granulosa cells were cultured in DMEM/F-12 media supplemented with exosome free fetal bovine serum (Exo-FBS). Once reached 70-75% confluency, cells were treated with 5 μM H<sub>2</sub>O<sub>2</sub> for 40 min. Phenotype and genotype analyses were performed on collected granulosa cells and isolated exosomes 24 hr post H<sub>2</sub>O<sub>2</sub> treatment. The second experiment was conducted on bovine preimplantation embryos produced in vitro under different culture conditions. For that, cumulus oocyte complexes (COCs) were aspirated from follicles 2-8 mm and matured in vitro. After in vitro fertilization of matured oocytes, zygotes were cultured in different culture media supplemented with or without epidermal growth factor (EGF) and/or hyaluronic acid (HA) at different developmental stages (Chapter 3, Figure 3.1). Samples were collected at blastocyst stage and mRNA expression and DNA methylation patterns of focal adhesion genes were analyzed. Both experiments were conducted on follicular cells and preimplantation embryos generated from slaughterhouse collected ovaries and thus special ethical approval for this experiment was not required.

#### 1.3.2 Granulosa cells phenotype measurements

All granulosa cell experimental groups were subjected to ROS accumulation level assay using 2', 7'-dichlorofluorescein diacetate (H2DCFDA) (Life Technologies, Germany) according to manufacturer's instructions. Images were captured using inverted fluorescence microscope (Leica DM IRB, Leica, Wetzlar, Germany) and analyzed using ImageJ 1.48v (National Institutes of Health, USA, <http://imagej.nih.gov>). Mitochondrial activity was measured using MitoTracker red dye (MitoTracker1 Red CMXRos, M7512; Invitrogen). For that, cells were incubated with 200 nM of MitoTracker red dye

for 30 min. Cells were fixed with 4% paraformaldehyde overnight at 4 °C and mounted with Vectashield (H-1200) containing DAPI. Images were acquired under confocal microscope CLSM LSM-780 and analyzed with ImageJ 1.48v (National Institutes of Health, USA, <http://imagej.nih.gov>). Cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technology, Japan) and the number of living cells was measured at a wave length of 450 nm using a microplate reader (BioTek Instruments Inc, Germany). Flow cytometry was used to investigate granulosa cell cycle profile. For that, granulosa cells from each experimental group were fixed and then stained with 50 µg/mL of propidium iodide (PI) and 50 µg/mL of RNase. Thereafter, cell cycle analysis was performed using BD LSRFortessa™ Flow cytometer (BD Biosciences) and data were analyzed using ModFit LT software (<http://www.vsh.com/products/mflt/index.asp>).

### 1.3.3 Blastocysts phenotype measurements

Developmental data for all blastocyst experimental groups were recorded. The total cell number in the blastocysts was determined using Hoechst 33342 stain (Sigma, Munich; Germany). Day 7 blastocysts cultured in basic media or supplemented with EGF and/or HA were subjected to ROS accumulation assay. Moreover, TUNEL assay kit (Roche®, Mannheim, Germany) was used to detect dead cells in blastocysts of different treatment group. The samples were observed under the fluorescence microscope (Leica, Germany) and TUNEL positive (fragmented DNA) cells were recorded. Furthermore, cryotolerance test was performed to assess the effect of culture media supplemented with EGF and/or HA on the freezability of produced blastocysts. The expansion and hatching rates were recorded at 24, 32, 48 and 56 h post thawing.

### 1.3.4 Exosomes isolation, characterization and labeling

Exosomes were isolated from granulosa cells culture media of both control and H<sub>2</sub>O<sub>2</sub>-challenged groups using an ultracentrifugation protocol. For that, culture media were collected 24 h post treatment and subjected to 10 min centrifugation at 300 g to discard cells followed by 3000 g for 30 min to remove dead cells and 10000 g for 30 min to discard cellular debris. The supernatant was centrifuged at 30000 g for 30 min to remove micro vesicles. Thereafter, exosomes were isolated using ultracentrifugation at 120,000 g for 70 min in a Beckman SWTi55 rotor. All centrifugation steps were performed at 4 °C. Identity and purity of isolated exosomes were assessed using



immunoblotting analysis of marker proteins (CD63, ALIX and CYCS). Size and concentration measurements of pure exosome samples were performed using the NanoSight NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK). Moreover, 30  $\mu$ L drops of purified exosomes on parafilm were used for exosomes characterization under electron microscope (Zeiss EM 109, Carl Zeiss, Germany). Thereafter, exosomes were subjected to mRNA analysis of candidate genes. In order to assess the uptake of exosomes by granulosa cells, the purified exosomes were labeled using PKH67 dye (Sigma, Germany) and co-incubated with granulosa cells and then cells were fixed in 4% paraformaldehyde overnight at 4 °C. After washing 2 times with PBS-CMF, cells were mounted in mounting medium containing DAPI and pictures were captured under confocal microscope (LSM780-Carl Zeiss, Carl Zeiss GmbH; Germany).

### 1.3.5 Total RNA isolation and gene expression analysis

Total RNA was isolated from granulosa cells and isolated exosomes using miRNeasy mini kit (Qiagen, Germany) following the manufacturer. However, Total RNA was isolated from blastocysts using RNA/DNA/protein purification plus micro kit (Norgen biotek, Cat# 51600, Canada) according to manufacturer's instructions. Total RNA was subjected to cDNA synthesis using first strand cDNA synthesis kit (Thermo Scientific, USA) and the relative mRNA expression was determined using quantitative real time PCR (qRT-PCR). The data were analyzed using a comparative threshold cycle ( $\Delta\Delta$ CT) method and expression levels of GAPDH and ACTB genes were used for normalization.

### 1.3.6 Protein quantification and localization

Western blotting was performed to quantify proteins in both experiments. For that, proteins were separated on SDS-PAGE gel and then transferred to nitrocellulose membranes (Protran®, Schleicher & Schuell Bioscience) using blotting apparatus. On the other hand, immunocytochemistry and immunohistochemistry were used to localize candidate proteins and pictures were captured under confocal microscope and analyzed using CLSM LSM-780 confocal laser scanning microscope (Zeiss, Germany). The images were analysed with ImageJ 1.48v (National Institutes of Health, USA, <http://imagej.nih.gov>). Details of all primary and secondary antibodies used through the study were mentioned in the respective chapters.

### 1.3.7 DNA extraction and bisulphite sequencing

DNA methylation pattern of focal adhesion pathway candidate genes was performed using DNA extracted from blastocyst samples used for mRNA expression analysis. For that, DNA was extracted using RNA/DNA/protein purification plus micro kit (Norgen biotek, Cat# 51600, Canada) according to the manufacturer's instructions. The extracted gDNA was subjected to bisulfite treatment using EZ DNA methylation direct kit (Zymo Research) and amplified using gene specific primers designed for bisulfite sequencing. PCR product was purified using the QIAquick PCR purification (Qiagen), cloned to pGEM®-T Easy Vector Systems (Promega, WI, USA) and transformed to *E. coli* competent cells. Thereafter, the bacteria were then plated onto the LB agar/ampicillin/IPTG/X-gal plate and incubated overnight at 37 °C. Independent colonies were collected and subjected to PCR amplification using M13 primer and sequenced using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter). Sequencing data were analyzed using the bisulfite sequencing DNA methylation analysis software (BISMA) (<http://services.ibc.uni-stuttgart.de/BDPC/BISMA/>).

### 1.3.8 Statistical analysis

Data of four independent biological replicates were subjected to statistical analysis and presented as mean  $\pm$  SEM. In the first experiment data were analyzed using GraphPad Prism (Version 5) and a two-tailed Student's t-test was used to compare the control and H<sub>2</sub>O<sub>2</sub>-challenged granulosa cells treatment groups while two-way analysis of variance (ANOVA) followed by multiple pair-wise comparisons using Tukey post-hoc test was performed to test the differences in the mean values between controls and exosomes co-incubated groups. Statistical differences among means in the second experiment were analyzed using Statistical Analysis System (SAS) version 9.1 software (SAS Institute Inc., Cary, NC, USA) and one-way ANOVA followed by multiple pairwise comparisons was performed. Differences were considered significant when the  $P < 0.05$ .

## 1.4 Results

The results are presented in detail with illustrated figures and tables in the respective chapters of this dissertation; however, the major findings are pointed out here.

### 1.4.1 Oxidative stress induced by H<sub>2</sub>O<sub>2</sub> induced mRNA and protein expression levels of Nrf2 and downstream antioxidant genes in bovine granulosa cells

In the first experiment (chapter 3), exposure of bovine granulosa cells to oxidative stress conditions induced by H<sub>2</sub>O<sub>2</sub> resulted in high ROS level accompanied with low mitochondrial activity compared to control group. Moreover, H<sub>2</sub>O<sub>2</sub>-challenged granulosa cells exhibited high mRNA expression level of Nrf2 with low expression level of Keap1 genes. Moreover, mRNA expression level of Nrf2 antioxidant downstream genes (PRDX1, HMOX1, CAT, SOD1, TXN1 and NQO1) tended to be higher under oxidative stress conditions. Immunoblotting and immunocytochemistry analysis revealed that, protein expression level of Nrf2 and CAT was found to be significantly higher in granulosa cells exposed to oxidative stress conditions compared with control ones.

### 1.4.2 Exosomal RNA was enriched with Nrf2 and selective antioxidant molecules in exosomes released under oxidative stress conditions

Nanoparticle tracking analysis showed that high concentration with large size was detected in exosomes that released under oxidative stress conditions (StressExo). Moreover, mRNA of these exosomes was enriched with Nrf2, CAT and TXN1. However, lower mRNA level of PRDX1, SOD1 and HMOX1 was detected in StressExo compared to the control group (NormalExo). Interestingly, mRNA of NQO1 was not detected in exosomal RNA of both exosome groups.

### 1.4.3 Exosomes horizontally transferred their mRNA contents after co-incubation with granulosa cells

Bovine granulosa cells cultured under oxidative stress or normal conditions were co-cultured with StressExo or NormalExo and the uptake of exosomes by granulosa cells was confirmed by confocal microscope. Results revealed that, co-culture of granulosa cells with StrssExo under normal or oxidative stress conditions increased mRNA and protein expression level of Nrf2 and downstream antioxidant molecules (PRDX1, CAT, HMOX1 and TXN1). These findings were in agreement with a reduction of intracellular

ROS level accompanied with high mitochondrial activity that were detected in granulosa cells cultured under oxidative stress conditions. Moreover, co-culture with StressExo resulted in higher proportion of cells arrested at G<sub>0</sub>/G<sub>1</sub> phase and a lower proportion in G<sub>2</sub>/M which was confirmed by increasing cell proliferation rate of granulosa cells under oxidative stress conditions.

#### 1.4.4 In vitro culture media supplemented with EGF and/or HA induced mRNA and protein expression levels of genes involved in focal adhesion pathway

Relative mRNA expression analysis revealed that, blastocysts derived from zygotes supplemented with EGF and/or HA exhibited higher mRNA expression level of genes involved in focal adhesion pathway (COL1A2, COL4A1, FAK, VCL, PTEN, RAC1, PAK4) compared to those cultured in basic media. HA and in a combined with EGF had the higher mRNA expression level. Protein analysis indicated that the protein level of FAK and VCL was in the same line with their mRNA expression level in each experimental group. Immunohistochemistry of VCL protein showed high cell membrane localization of VCL protein with the presence of HA or in a combination with EGF.

#### 1.4.5 The supplementation with EGF and/or HA pre or post embryonic genome activation altered the expression of genes involved in focal adhesion pathway

To investigate the effective time point of culture media supplemented with EGF and HA on the alteration of focal adhesion pathway, zygotes were cultured at different developmental stages before or after EGA in media supplemented with or without EGF and HA. Results demonstrated that high mRNA expression level of genes involved in focal adhesion pathway was found with the presence of EGF and HA during EGA period (Until\_16C) or (After\_4C).

#### 1.4.6 DNA methylation mediated the regulation of genes involved in focal adhesion pathway of embryos supplemented with EGF and/or HA

According to mRNA and protein expression levels of focal adhesion pathway as well as embryo development and quality of blastocysts derived from embryos that were cultured with the supplementation of EGF and/or HA; we hypothesized that different culture conditions (BM, +EGF, +HA and +EGF+HA) affect embryo development and quality through epigenetic regulation of genes involved in focal adhesion pathway.

DNA methylation is considered one of the main common epigenetic regulatory mechanisms that control gene expression. Here, the mRNA expression of genes responsible of DNA maintenance (DNMT1) and de novo methylation (DNMT3A and DNMT3B) revealed that, the culture media supplemented with EGF and/or HA at different developmental stages dysregulated the mRNA expression level of DNA methyltransferases genes (DNMT1, DNMT3A and DNMT3B). Moreover, culture media supplemented with EGF and/or HA until different developmental stages altered the DNA methylation pattern at promoter, distal promoter or gene body of COL1A2, COL4A1 and RAC1 genes of produced blastocysts. The bisulfite sequencing results indicated that the DNA methylation pattern of COL1A2 and COL4A1 at the promoter region was negatively correlated with the gene expression patterns in blastocysts of After\_4C, After\_16C, Until\_4C and BM+HA groups. However, the DNA methylation level in the distal promoter region of COL1A2 was positively correlated with its gene expression in all blastocysts groups except in After\_4C and After\_16C groups. Moreover, the DNA methylation pattern at the intronic region of the RAC1 was positively correlated with gene expression in all blastocyst groups except After\_16C; BM + EGF and BM + HA groups.

In conclusion, cells response to suboptimal culture conditions such as oxidative stress and culture media via the activation of cell-cell communication mechanisms mediated by exosome secretion and epigenetic regulation of focal adhesion pathway which subsequently alter cellular functions and embryo development and quality.

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Chapter 2: Exosomes mediated cell-cell communication under oxidative stress response  
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**Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress**

Mohammed Saeed-Zidane<sup>1</sup>, Lea Linden<sup>1</sup>, Dessie Salilew-Wondim<sup>1</sup>, Eva Held<sup>1</sup>, Christiane Neuhoff<sup>1</sup>, Ernst Tholen<sup>1</sup>, Michael Hoelker<sup>1,2,3</sup>, Karl Schellander<sup>1,2</sup>, Dawit Tesfaye<sup>1,2</sup>

<sup>1</sup>Institute of Animal Science, Department of Animal Breeding and Husbandry, University of Bonn, Bonn, Germany

<sup>2</sup>Teaching and Research Station Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany

<sup>3</sup>Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

\*Correspondence to:

Dawit Tesfaye, PhD

Department of Animal Breeding and Husbandry

Institute of Animal Science

Endenicher Allee 15, 53115 Bonn, Germany.

E-mail: [tesfaye@itw.uni-bonn.de](mailto:tesfaye@itw.uni-bonn.de)

Short title: Oxidative stress response in bovine granulosa cells

## 2.1 Abstract

Various environmental insults including diseases, heat and oxidative stress could lead to abnormal growth, functions and apoptosis in granulosa cells during ovarian follicle growth and oocyte maturation. Despite the fact that cells exposed to oxidative stress are responding transcriptionally, the potential release of transcripts associated with oxidative stress response into extracellular space through exosomes is not yet determined. Therefore, here we aimed to investigate the effect of oxidative stress in bovine granulosa cells in vitro on the cellular and exosome mediated defense mechanisms. Bovine granulosa cells were aspirated from ovarian follicles and cultured in DMEM/F-12 Ham culture medium supplemented with 10% exosome-depleted fetal bovine serum. In the first experiment sub-confluent cells were treated with 5  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 40 min to induce oxidative stress. Thereafter, cells were subjected to ROS and mitochondrial staining, cell proliferation and cell cycle assays. Furthermore, gene and protein expression analysis were performed in H<sub>2</sub>O<sub>2</sub>-challenged versus control group 24 h post-treatment using qRT-PCR and immune blotting or immunocytochemistry assay, respectively. Moreover, exosomes were isolated from spent media using ultracentrifugation procedure, and subsequently used for RNA isolation and qRT-PCR. In the second experiment, exosomes released by granulosa cells under oxidative stress (StressExo) or those released by granulosa cells without oxidative stress (NormalExo) were co-incubated with bovine granulosa cells in vitro to proof the potential horizontal transfer of defense molecules from exosomes to granulosa cells and investigate any phenotype changes. Exposure of bovine granulosa cells to H<sub>2</sub>O<sub>2</sub> induced the accumulation of ROS, reduced mitochondrial activity, increased expression of Nrf2 and its downstream antioxidant genes (both mRNA and protein), altered the cell cycle transitions and induced cellular apoptosis. Granulosa cells exposed to oxidative stress released exosomes enriched with mRNA of Nrf2 and candidate antioxidants. Subsequent co-incubation of StressExo with cultured granulosa cells could alter the relative abundance of cellular oxidative stress response molecules including Nrf2 and antioxidants CAT, PRDX1 and TXN1. The present study provides evidences that granulosa cells exposed to oxidative stress conditions react to stress by activating cascades of cellular antioxidant molecules which can also be released into extracellular environment through exosomes.

**Keywords:** Exosomes, Oxidative stress, Granulosa cells, Antioxidants.

## 2.2 Introduction

Stress induced by environment or physiology of the animals is considered as one of the important causes of impaired fertility in the dairy cattle (Abuelo et al. 2015). A considerable number of evidences manifested that, various environmental and physiological insults including diseases, heat and oxidative stress could lead to abnormal growth and function of granulosa cells in ovarian follicular development (Li et al. 2016, Zhang et al. 2016). Subsequently, granulosa cells apoptosis is responsible for follicular atresia (Hickey et al. 1988) and subsequently oocyte and ovarian dysfunction (Lee et al. 2013, Chaube et al. 2016). Oxidative stress is defined as imbalance between the level of intracellular ROS production including superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $^{\cdot}OH$ ) and their scavenge by antioxidants (Betteridge 2000, Burton and Jauniaux 2011, Schieber and Chandel 2014). Although  $^{\cdot}OH$  is the most harmful free radical,  $H_2O_2$  has long half-life than the other free radicals which allowed a longer reaction with all of the cellular components including DNA. Therefore, despite lower reactivity of  $H_2O_2$ , its relatively longer half-life provides enough time for the molecule to move into the nucleus of the cell (Aprioku 2013). Despite the fact that cells exposed to oxidative stress respond transcriptionally (Kops et al. 2002, Omata et al. 2008, Szybowska et al. 2011), the role of extracellular vesicles including exosomes in mediating cell's response to oxidative stress should be carefully ruled (Eldh et al. 2010).

Direct or indirect interactions of mammalian gametes with the surrounding somatic cells including granulosa and theca cells is vital for successful folliculogenesis (Vanderhyden et al. 1990, Vanderhyden et al. 1992, Eppig et al. 2002, Matzuk et al. 2002). The bidirectional communication between oocyte and surrounding cells during follicular development (Kidder and Vanderhyden 2010) can be mediated by extracellular vesicles (Sohel et al. 2013, Di Pietro 2016). Extracellular vesicles including exosomes (30-150 nm), microvesicles (150-1500) and apoptotic bodies (500-2000 nm) are derived from plasma membrane, outward budding of plasma membrane and outward blebbing of apoptotic cell membrane, respectively (El Andaloussi et al. 2013). Exosomes are nano-sized vesicles and a member of extracellular membrane vesicles which mediate cell-to-cell communication under various conditions (da Silveira et al. 2012, Sohel et al. 2013, Ciardiello et al. 2016). Furthermore, they are able to carry different cytosolic

macromolecules (mRNA, miRNA and proteins) which can be transferred to recipient cells and induced alterations in their physiological functions (Di Pietro 2016).

Exosomes are part of extracellular vesicles with a size of 30-150 nm (Li et al. 2014, Sharma et al. 2016) that released through exocytosis process to the extracellular space and found in various biological fluids (Pan et al. 1985, Théry et al. 2002, Keller et al. 2006, Sluijter et al. 2014, Frydrychowicz et al. 2015). Regardless of origin, exosomes have similar and specific surface proteins such as CD9, CD63, CD81 and Alix (Vlassov et al. 2012). In fact, exosomes contain a cargo of nucleic acids (DNA, mRNA, ncRNA), proteins, lipids and other molecules (Silva and Melo 2015) and play vital role in cell-to-cell communication resulting in physiological changes in recipient cells (Valadi et al. 2007, Peinado et al. 2012). Various cell types have been shown to release exosomes with various diversity in quality and quantity into the extracellular space as a response to various environmental insults and different pathological conditions (Eldh et al. 2010, Yuan et al. 2016). Especially exosomes released under oxidative stress conditions could mediate a signal to recipient cells that alter their defense mechanism to prevent cell death under oxidative stress conditions (Eldh et al. 2010). Therefore, extracellular vesicles have been regarded as signalosomes: multifunctional signaling complexes for regulating fundamental cellular and biological functions (El Andaloussi et al. 2013).

Therefore, we hypothesized that granulosa cells under oxidative stress respond via activation of the cellular Nrf2 and downstream antioxidant molecules and those molecules will be released into extracellular space through exosomes. To proof this hypothesis a granulosa cell culture system was used as a model to investigate the response of cells to oxidative stress induced by H<sub>2</sub>O<sub>2</sub>. Results demonstrated the significant effect on granulosa cells ROS level, mitochondrial activity, proliferation, differentiation and cell cycle. Moreover, exosomes released from granulosa cells under oxidative stress conditions into extracellular space were investigated for the presence of antioxidant molecules as molecular responses to cellular stress.

## 2.3 Material and methods

### 2.3.1 Experimental design

To determine the right concentration of H<sub>2</sub>O<sub>2</sub> that induces ROS accumulation without a deleterious effect on granulosa cells, different doses of H<sub>2</sub>O<sub>2</sub> (2.5, 5, 10, 20 and 50 µM) was used to treat in vitro cultured bovine granulosa cells. Depending on the various

investigations including morphological evaluation, ROS staining, mitochondrial activity and cell viability assays which indicated as supplemental figure, a 5  $\mu\text{M}$   $\text{H}_2\text{O}_2$  was selected as moderate oxidative stress inducer in cultured granulosa cells in the present study. The first experiment was conducted to assess the effect of oxidative stress on granulosa cell functions as well as their cellular and extracellular response with regard to antioxidant molecules. For that, bovine granulosa cells were aspirated from small follicles (3-8 mm) and cultured in DMEM/F-12 Ham culture medium supplemented with 10% exosome-depleted fetal bovine serum (System Biosciences, USA). Sub-confluent cells were exposed to 5  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 40 min. Twenty four hours post-treatment intracellular ROS level, mitochondrial activity, cell proliferation and the cell cycle assays were performed. Moreover, the cellular mRNA and protein expression levels were quantified. The spent media of the same cultured granulosa cells was collected for exosome isolation and subsequent analysis of transcript abundance for Nrf2 and its antioxidant downstream genes. The second experiment was carried out to elucidate the potential horizontal transfer of oxidative stress related molecular signal carried by exosomes from donor to recipient cells. For that, sub-confluent granulosa cells were co-cultured (with or without  $\text{H}_2\text{O}_2$ ) with exosomes derived from spent media of stressed granulosa cells (StressExo) or from spent media of granulosa cells without stress (NormalExo). All phenotypic and molecular changes in recipient cells were investigated to proof the effect of exosome mediated horizontal transfer of defence molecules in recipient cells.

### 2.3.2 Bovine granulosa cell culture

Bovine ovaries were collected from a local abattoir and transported within 1-2 h to the lab in a thermic flask containing physiological saline solution (0.9% NaCl) at 37 °C. Upon arrival, ovaries were washed 2-3 times with 37 °C 0.9% NaCl, followed by rinsing in 70% warm ethanol for 30 sec and washed 3 times with 0.9% NaCl. Granulosa cells were aspirated from small growing follicles (3-8 mm diameter) using 18-gauge sterile needle and transferred into a 15 mL sterilized falcon tube containing  $\text{Ca}^{2+}/\text{Mg}^{2+}$  free 1x phosphate buffer saline (PBS-CMF). The cumulus-oocyte-complexes (COC) were left to stabilize at the bottom of the tube under 37 °C. The upper phase containing granulosa cells was carefully transferred into new tubes with 1x PBS-CMF and centrifuged at 750 g for 7 min. The granulosa cell pellets were re-suspended in 500  $\mu\text{l}$

red blood cell (RBC) lysis buffer for 1 min, followed by adding 3 mL DMEM/F-12 Ham (Sigma, Germany) and centrifuged at 500 g for 5 min. Afterwards, the pellets were washed with DMEM/F-12 Ham culture media supplemented with 10% exosome-depleted fetal bovine serum (System Biosciences, USA), 100 IU/mL penicillin and 100 µg/mL of streptomycin (Sigma, Germany), and 100 µg/mL fungizone (Sigma, Germany). Cell viability and concentration were determined using trypan blue exclusion method. Finally, a total of  $2.5 \times 10^5$  live cell per well were seeded into CytoOne<sup>®</sup> 24-well plate (Starlab International GmbH, Germany) in 600 µL DMEM/F-12 Ham culture media supplemented with 10% exosome free FBS and incubated at 37 °C with 5% CO<sub>2</sub>.

### 2.3.3 ROS detection

Intracellular ROS level was determined using 2', 7'-dichlorofluorescein diacetate (H2DCFDA) (Life Technologies, Germany) according to the manufacturer's instructions with some modifications. Briefly, granulosa cells from each group were cultured in 96-well plate and then incubated with 50 µl of 75 µM H2DCFDA for 20 min in dark at 37 °C. Afterwards, incubated cells were washed twice with (PBS-CMF) and images were captured under inverted fluorescence microscope (Leica DM IRB, Leica, Wetzlar, Germany) using a green-fluorescence filter and images were analyzed using ImageJ 1.48v (National Institutes of Health, USA, <http://imagej.nih.gov>).

### 2.3.4 Mitochondrial staining

Approximately  $3 \times 10^4$  live cells per well were cultured in 8-well slide chamber and subjected to mitochondrial activity assay. For this, cells were incubated with 200 nM of MitoTracker red dye (MitoTracker1 Red CMXRos, M7512; Invitrogen) for 30 min. After washing twice with PBS-CMF, cells were fixed with 4% paraformaldehyde overnight at 4 °C. Fixed cells were mounted with Vectashield (H-1200) containing DAPI. Images were acquired under confocal microscope CLSM LSM-780 and analyzed with ImageJ 1.48v (National Institutes of Health, USA, <http://imagej.nih.gov>).

### 2.3.5 Cell proliferation assay

Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technology, Japan) was used in the present study to perform cell proliferation assay. Briefly,  $1 \times 10^4$  live cells per well were seeded in 96-well plate and according to the manufacturer's instructions 10 µl of CCK-8/well was added to sub-confluent cells from all experimental groups, and then

incubated for 3 h at 37 °C in 5% CO<sub>2</sub>. The optical density (OD) of released formazan dye which an indirect indicator of the number of living cells was measured at a wave length of 450 nm using a microplate reader (BioTek Instruments Inc, Germany). The OD of wells that contained only culture media was used as blank for normalization purpose.

### 2.3.6 Cell cycle assay

Flow cytometry was used to demonstrate cell cycle profile in granulosa cells from each group under study. For that, cultured cells were trypsinized and centrifuged at 750 g for 5 min followed by two times washing with PBS-CMF. Cells were counted and a minimum  $1 \times 10^6$  of live cells were fixed in 70% ice-cold ethanol overnight at 4 °C. The fixed cells were then centrifuged at 1200 g for 5 min, and the pellets were re-suspended and washed twice with 500 µL of PBS-CMF. Thereafter, cells were stained with 50 µg/mL of propidium iodide (PI) and 50 µg/mL of RNase and kept at 37 °C for 30 min. Cell cycle analysis was performed using BD LSRFortessa™ Flow cytometer (BD Biosciences). Data were analyzed using ModFit LT software (<http://www.vsh.com/products/mflt/index.asp>).

### 2.3.7 Exosome isolation

Spent culture media from control and H<sub>2</sub>O<sub>2</sub>-challenged groups were collected and subjected to exosomes isolation procedure. Briefly, collected spent media were centrifuged at 300 g for 10 min to discard cells and 3000 g for 30 to remove dead cells and 10000 g to discard cellular debris followed by 30 min centrifugation at 30000 g to remove micro vesicles. After that, exosomes were isolated using ultracentrifugation at 120,000 g for 70 min in a Beckman SWTi55 rotor. Exosomes were washed with PBS-CMF and centrifuged once more at 120,000 g for 70 min. All centrifugation steps were performed at 4 °C unless indicated. Finally, isolated exosomes were suspended in PBS-CMF and stored at -80 °C for further applications.

### 2.3.8 Nanoparticle tracking and electron microscopy analysis

Exosomes identity and purity were determined by immune blotting of exosomal and cellular marker proteins (CD63, Alix and CYCS). Concentration and size distribution of exosomes was performed using NanoSight NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK). Briefly, 10 µl of purified exosomes were diluted



in 1 mL PBS-CMF and used for five recording videos, videos were analyzed to give mean, mode, standard deviation and concentration of particles using NTA software. Moreover, electron microscope (Ziess EM 109, Carl Zeiss) was used for exosomes characterization, 30  $\mu$ l drops of purified exosomes on parafilm were used to be absorbed by Formvar/carbon-coated grids. Five minutes later the Formvar/carbon-coated grids were washed using drops of PBS before incubation with 30  $\mu$ l drops of 2% uranyl acetate. Grids were washed with drops of PBS then examined and captured under electron microscope.

### 2.3.9 RNA extraction and cDNA synthesis

Total RNA was isolated from collected cells and isolated exosomes using the miRNeasy Mini kit (Qiagen, Hilden; Germany) according to manufacturer's protocol including DNase digestion for removal of possible genomic DNA contamination. RNA concentration was measured using NanoDrop 8000 spectrophotometer (NanoDrop technologies). The cDNA was synthesized from total RNA using first stand cDNA synthesis kit (Thermo Fisher scientific, Germany). RNA concentration was adjusted using RNase free water and a maximum volume of 10  $\mu$ l RNA from each replicate was co-incubated with 0.5  $\mu$ l of 100  $\mu$ M Oligo (dT)<sub>18</sub> and 0.5  $\mu$ l of Random Primer at 65 °C for 5 min then chilled on ice for 2 min. Thereafter, 1  $\mu$ l RiboLock RNase Inhibitor, 4  $\mu$ l 5x Reaction Buffer, 2  $\mu$ l dNTP and 2  $\mu$ l RevertAid Reverse Transcriptase, were added and incubated at 25 °C for 5 min, 37 °C for 60 min and 70 °C for 5 min then subjected to gene expression analysis.

### 2.3.10 Quantitative RT-PCR analysis of selected candidate genes

Relative transcript abundance of oxidative stress response genes (Nrf2, Keap1, SOD1, CAT, PRDX1, HMOX1, TXN1 and NQO1) was quantified using cDNA generated from cultured granulosa cells and isolated exosomes. Moreover, cell proliferation related genes (CCDN2 and PCNA), cell differentiation related genes (CYP11A1 and STAR1) and proapoptotic (Casp3) and antiapoptotic (BCL2L1) related genes were quantified only in granulosa cells using quantitative real time PCR in Applied Biosystem® StepOnePlus™ System (Thermo Fisher Scientific, Germany), using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Germany). The real time PCR was run using the following program: 95 °C for 3 min, 40 cycles at 95 °C for 15 sec, 60 °C for 45 sec followed by melting curve analysis. Data were analyzed using

comparative threshold cycle method ( $\Delta\Delta\text{CT}$ ) using actin, beta (ACTB) and phosphate dehydrogenase (GAPDH) as internal controls for cellular mRNA and ACTB, GAPDH and 18S genes for exosomal mRNA. All Primers listed in Supplementary Table 1 were designed using primer designing tool online software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

### 2.3.11 Western blotting

Protein expression of (ACTB, Nrf2, Keap1, CAT, StAR1, PCNA) proteins in granulosa cells and (CYCS, CD63 and Alix) proteins in isolated exosomes were performed using immunoblotting. Isolated cellular protein and exosomes from each group were boiled with 2x SDS loading buffer at 95 °C for 5 min before loading on a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Protran®, Schleicher & Schuell Bioscience) using blotting apparatus adjusted to 84 mA for 55 min. Membranes were blocked at room temperature with Roti-block solution (Carl Roth GmbH) for 1 h and then incubated over night at 4 °C with primary antibody against each of the candidate cellular proteins (Santa Cruz Biotechnology Inc, Germany: mouse monoclonal  $\beta$ -Actin (1:500), rabbit Polyclonal Nrf2 (1:200), Keap1 (1:300), StAR1 (1:350), PCNA (1:350) and 1: 300 rabbit polyclonal CAT, Life span Biosciences, Inc. Germany) or exosomal marker proteins (Santa Cruz Biotechnology Inc, Germany: goat polyclonal CYCS (1:350), Alix (1:350) and 1:250 rabbit polyclonal CD63 (System BioSciences, USA). Afterwards, the membranes were washed with Tween-Tris-buffer saline (TTBS) and then incubated with secondary antibody (Santa Cruz Biotechnology Inc, Germany: goat anti mouse (1:5000), goat anti rabbit (1:5000) and donkey anti goat (1:5000) for 1 h at room temperature. Thereafter, the membranes were incubated with equal amount of peroxide solution and luminol\enhancer at room temperature for 5 min in dark. Images were developed on ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany).

Table 2.1. The list of primers and their sequences of selected candidate genes used for qRT-PCR analysis

Gene	Primer sequence	Size(bp)	Accession Nr
GAPDH	F: 5'-ACCCAGAAGACTGTGGATGG-3' R: 5'-ACGCCTGCTTCACCACCTT-3'	247	NM_001034034
B-ACTIN	F: 5'-GGCATTACGAAACTACCTT-3' R: 5'-CAATCCACACGGAGTACTTG-3'	208	NM_173979
18S	F: 5'-CGCAGCTAGGAATAATGGAA-3' R: 5'-TCTGATCGTCTTCGAACCTC-3'	210	NR_036642
NrF2	F: 5'-CCCAGTCTTCACTGCTCCTC-3' R: 5'-TCAGCCAGCTTGTCATTTTG-3'	165	NM_001011678
Keap1	F: 5'-TCACCAGGGAAGGATCTACG-3' R: 5'-AGCGGCTCAACAGGTACAGT-3'	199	NM_001101142.1
CAT	F: 5'-TGGGACCCAACCTATCTCCAG-3' R: 5'-AAGTGGGTCCTGTGTTCCAG-3'	178	NM_001035386.1
PRDX1	F: 5'-TGGATCAACACACCCAAGAA-3' R: 5'-GTCTCAGCGTCTCATCCACA-3'	217	NM_174431.1
SOD1	F: 5'-TGCCATCGTGGATATTGTAG-3' R: 5'-GCAATTCCAATTACACCACA-3'	174	NM_174615
HMOX1	F: 5'-CAAGGAGAACCCCGTCTACA-3' R: 5'-CCAGACAGGTCTCCCAGGTA-3'	225	NM_001014912
TXN1	F: 5'-AGCTGCCAAGATGGTGAAAC-3' R: 5'-ACTCTGCAGCAACATCCTGA-3'	215	NM_173968.3
NQO1	F: 5'-AACCAACAGACCAGCCAATC-3' R: 5'-CACAGTGACCTCCATCCTT-3'	154	NM_001034535.1
Casp3	F: 5'-TGCCACTGTATCAGGGAACA-3' R: 5'-TGCTCAGCACAAACATCACA-3'	185	NM_201107726
BCL2L1	F: 5'-AAGTTTTCTGACCCTTTTCC-3' R: 5'-GGTCCTGGTCCTTATTTCTT-3'	213	XM_015474117.1
CCND2	F: 5'-CGACTTCATCGAACACATCC-3' R: 5'-ATCTTTGCCAGGAGATCCAC-3'	234	NM_001076372.1
PCNA	F: 5'-CACCAGCATGTCCAAAATAC-3' R: 5'-CTGAGATCTCGGCATATACG-3'	240	NM_001034494.1
CYP11A1	F: 5'-CGGAAAGTTTGTAGGGGACA-3' R: 5'-ACGTTGAGCAGAGGGGACT-3'	177	NM_176644.2
STAR	F: 5'-AAATCCCTTTCCAAGGTCTG-3' R: 5'-ACCAGCATTCTGCTACTGC-3'	205	XM_006054485.1

### 2.3.12 immunocytochemistry

The cellular detection and localization of Nrf2 (Santa Cruz Biotechnology Inc, Germany, 1:200 polyclonal rabbit Nrf2) and CAT (1:250 polyclonal CAT, Life span Biosciences, Inc. Germany) proteins were determined using immunocytochemistry assay. For this, granulosa cells from each group were cultured in 4-well slide chamber and subjected to immunocytochemistry assay. Cells were fixed in 4% paraformaldehyde overnight at 4 °C. Fixed cells were then washed 3 times with PBS-CMF and subsequently incubated with 0.3% triton for 10 min followed by blocking with 3% donkey serum for 1 h at room temperature followed by incubation with primary antibody overnight at 4 °C. Following that, cells were washed 3 times with PBS-CMF and incubated with fluorescent secondary antibody (Santa Cruz Biotechnology Inc, Germany, Alexa flour goat anti rabbit 1:350) for 3 h at 37 °C. After washing twice with PBS-CMF, the cells were mounted in mounting medium containing DAPI. Images were taken under laser scanning confocal microscope (LSM780-Carl Zeiss, Carl Zeiss GmbH; Germany) and analyzed with ImageJ 1.48v (National Institutes of Health, USA, <http://imagej.nih.gov>).

### 2.3.13 Exosome labeling and co-incubation with cultured granulosa cells

The uptake of exosomes by cultured granulosa cells was assessed after co-incubation using confocal microscope (LSM780-Carl Zeiss, Carl Zeiss GmbH; Germany). For this, purified exosomes were labeled using PKH67 dye (Sigma, Germany) according to the manufacturer's instructions as described previously (Sohel et al. 2013). Briefly, exosomes were suspended in 1 mL of diluent C containing 5 µM PKH67 and incubated for 5 min. The labeling action was stopped by incubating for 1 min with an equal volume of exosome free FBS (system Biosciences, CA; USA) and then DMEM/F-12 media (Sigma, Germany) supplemented with 10% exosome free FBS was added and centrifuged at 120,000 g. Exosomes were washed two times with DMEM/F-12 and centrifuged at 120,000 g. Thereafter, exosomes were re-suspended in DMEM/F-12 media supplemented with 10% exosome free FBS. Granulosa cells were cultured in 8-well slide chamber in DMEM/F-12 media supplemented with 10% exosome free FBS and labeled exosomes were co-incubated with granulosa cells treated with or without H<sub>2</sub>O<sub>2</sub>. Cells were fixed in 4% paraformaldehyde overnight at 4 °C. After washing 2 times with PBS-CMF, cells were mounted in mounting medium containing DAPI and

the up-take of exosomes was confirmed under a laser scanning confocal microscope (LSM780-Carl Zeiss Carl Zeiss GmbH; Germany). Twenty four hours post-treatment cells were investigated for ROS level, mitochondrial activity, cell proliferation, cell cycle, protein analysis using immunoblotting and immunocytochemistry assays. Moreover, mRNA expression of Nrf2 and antioxidant downstream genes was performed after co-incubation granulosa cells with exosomes from stressed cells (StressExo) or exosomes from untreated normal cells (NormalExo).

#### 2.3.14 Statistical analysis

Data were analyzed using GraphPad Prism (Version 5) and presented as mean  $\pm$  SEM of four independent biological replicates. For response of granulosa cells to oxidative stress induced by H<sub>2</sub>O<sub>2</sub> in the first experiment, statistical differences in the mean values between treatment groups were compared using a two-tailed Student's t-test. Statistical differences among means in the second experiment were analyzed using two-way analysis of variance (ANOVA) followed by multiple pair-wise comparisons using Tukey post-hoc test. Statistical significance was defined at  $p \leq 0.05$ .

### 2.4 Results

#### 2.4.1 Dose dependent effect of H<sub>2</sub>O<sub>2</sub> on cultured bovine granulosa cells

In order to select a physiologically acceptable ROS inducer we have tested different doses of H<sub>2</sub>O<sub>2</sub> (2.5, 5, 10, 20 and 50  $\mu$ M) and subsequent morphological and physiological assessments were done to select the optimal dose of H<sub>2</sub>O<sub>2</sub> for further studies. As shown in supplemental file 1 morphological evaluation of granulosa cells after treatment revealed physical death of cells at concentrations beyond 5 micro molar of H<sub>2</sub>O<sub>2</sub>. Especially at doses beyond 50 micro molar of H<sub>2</sub>O<sub>2</sub> significant proportion of cells were found dead. In addition to that the ROS signal started to increase at 5  $\mu$ M H<sub>2</sub>O<sub>2</sub> compared to 2.5 and it does not change beyond the 5  $\mu$ M concentration (Suppl. Figure 2.2). Moreover, treatment of granulosa cells with all doses of H<sub>2</sub>O<sub>2</sub> except 2.5  $\mu$ M resulted in significant reduction in mitochondrial activity (Suppl. Figure 2.3). The 5  $\mu$ M dose is the minimum dose which resulted in significant reduction in mitochondrial activity compared to the other higher doses. Therefore, due to an induction of significant accumulation of ROS with moderate effect on morphology, mitochondrial activity and cell viability, a 5  $\mu$ M H<sub>2</sub>O<sub>2</sub> dose was selected to be used in the further experimental setups.

### 2.4.2 Effect of oxidative stress on cell morphology, ROS accumulation and mitochondrial activity in bovine granulosa cells

The H<sub>2</sub>O<sub>2</sub> treatment resulted in morphological changes associated with semi rounded shape and shrunken membrane (Figures 2.1A and B). Moreover, cells challenged with 5 μM H<sub>2</sub>O<sub>2</sub> exhibited significantly higher accumulation of intracellular ROS as compared to the untreated control at 24 hours post treatment (Figures 2.1C and D). On the other hand, the mitochondrial activity of H<sub>2</sub>O<sub>2</sub> challenged cells was lower than the untreated controls as illustrated in figure 2.2.

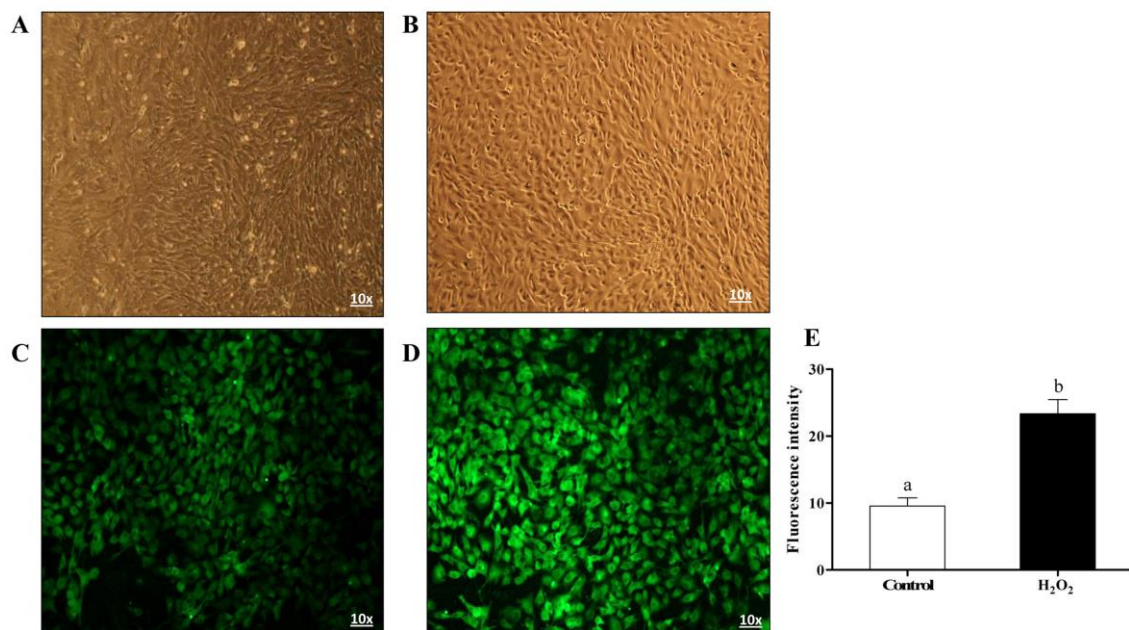


Figure 2.1: H<sub>2</sub>O<sub>2</sub> altered cell morphology and ROS accumulation in bovine granulosa cells. Cell morphological changes (A and B), intracellular ROS level (C and D) and ROS fluorescence intensity analysis (E) in H<sub>2</sub>O<sub>2</sub> untreated and treated groups, respectively. Data are mean ± SEM from four independent biological replicates. Bars with different letters (a, b) showed statistically significant differences ( $p < 0.01$ )

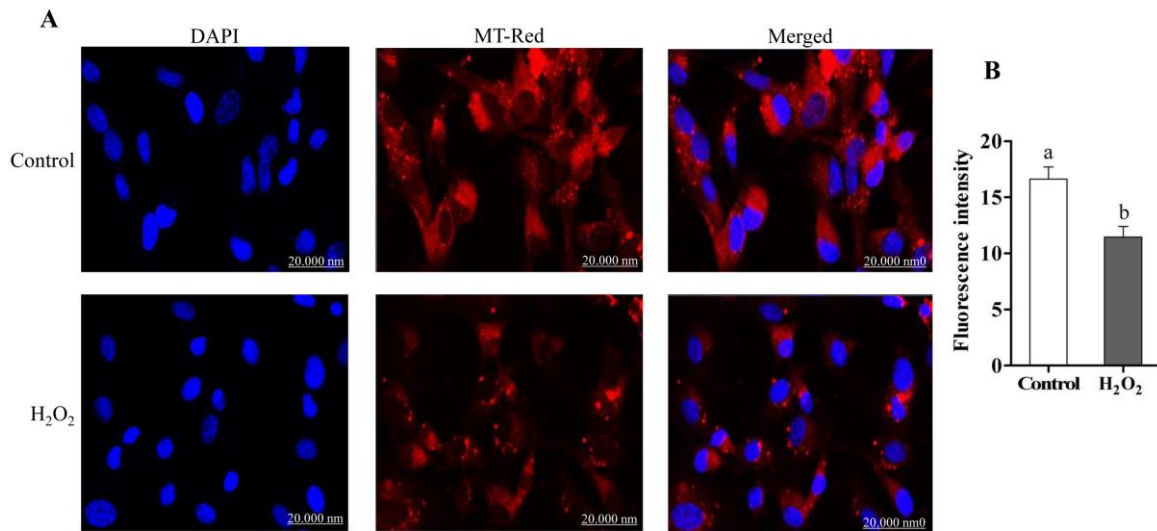


Figure 2.2: Lower level of mitochondrial activity in H<sub>2</sub>O<sub>2</sub>-treated cells compared to control group. The mitochondrial activity (A) and the fluorescence intensity analysis (B) in control and treated groups. The red colour indicated the MT-RED, while the blue colour indicated the nuclear staining using 4',6-diamidino-2-phenylindole (DAPI). Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b) showed statistically significant differences ( $p < 0.05$ )

#### 2.4.3 Oxidative stress reduced cell proliferation and G<sub>0</sub>/G<sub>1</sub> cell cycle transition

Cell proliferation assay results showed a reduction in viability of bovine granulosa cells challenged with 5  $\mu$ M H<sub>2</sub>O<sub>2</sub> compared to untreated controls (Figure 2.3A). These results were in agreement with cell cycle profile, which showed lower proportion of cells arrested at G<sub>0</sub>/G<sub>1</sub> phase (72.24%) in H<sub>2</sub>O<sub>2</sub> challenged cells as compared to the control ones (79.04%). Moreover, the percentage of cells that were found to be arrested at the G<sub>2</sub>/M phase was higher in cells challenged with 5  $\mu$ M H<sub>2</sub>O<sub>2</sub> compared to untreated controls (16.73% vs. 10.61%) (Figures 2.3B and C).

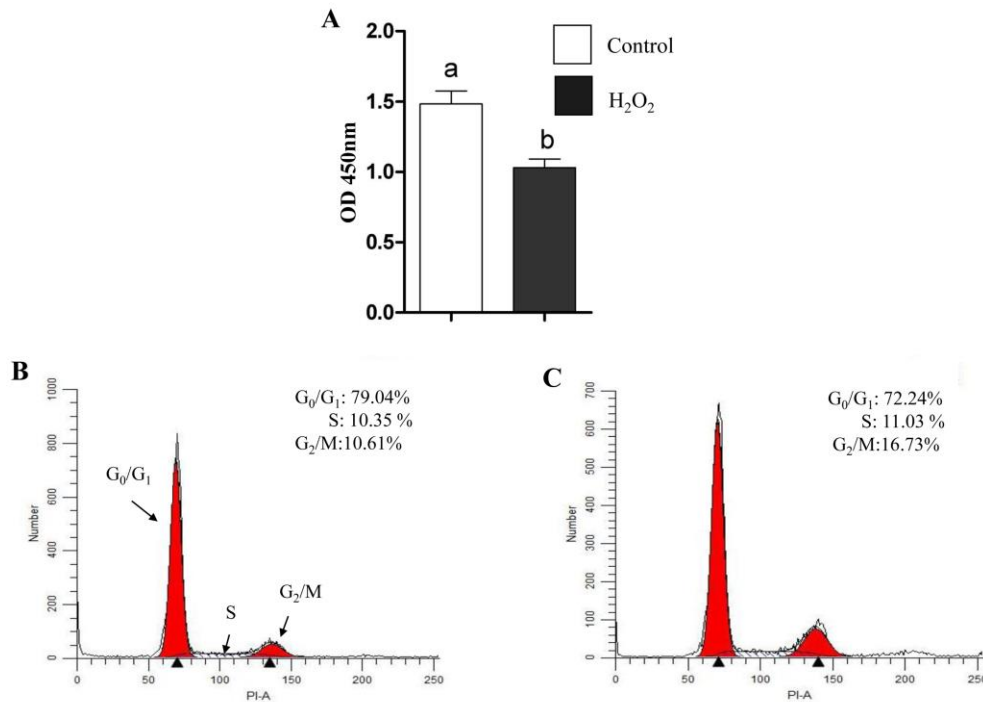


Figure 2.3: Oxidative stress resulted in reduced granulosa cell proliferation and a shift in cell cycle transition. (A) The proliferation rate in control (white bar) versus H<sub>2</sub>O<sub>2</sub> treated cells (black bar). Cell cycle analysis of granulosa cells under normal (B) or under oxidative stress conditions (C). The Y-axis indicated the cell count, while X-axis indicated the DNA content of cells detected by PI staining. Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b) showed statistically significant differences ( $p < 0.05$ )

#### 2.4.4 Oxidative stress increased the mRNA and protein expression levels of Nrf2 and its downstream antioxidants

Significantly higher mRNA and protein expression levels of Nrf2 was accompanied by lower mRNA and protein levels of its inhibitor Keap1 in granulosa cells challenged with H<sub>2</sub>O<sub>2</sub> compared to those cultured under normal conditions (Figures 4, 5A and 5B). Moreover, cells challenged with oxidative stress showed significantly increased expression level of NRF2 downstream antioxidants namely: PRDX1 and TXN1. However, despite an elevated expression of mRNA for SOD1, CAT, HMOX1 and NQO1 antioxidants in challenged granulosa cells but those differences were not statistically significant (Figure 2.4). In contrary to that immunoblotting and immunocytochemistry assays revealed a significantly higher level of CAT protein in granulosa cells under oxidative stress conditions (Figures 2.5A and 2.5C).



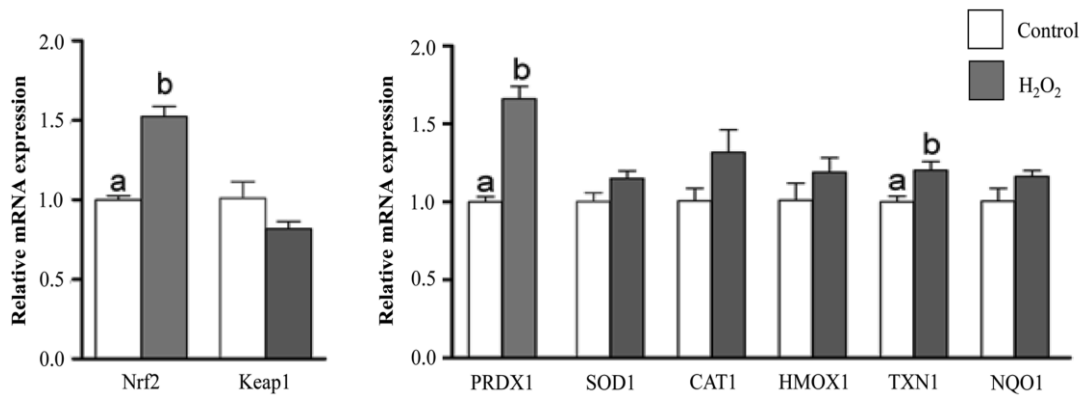


Figure 2.4: Oxidative stress increased cellular mRNA expression level of Nrf2 and its downstream antioxidants. qRT-PCR analysis of Nrf2 and downstream antioxidant genes in granulosa cells under normal (white bar) or oxidative stress (dark bar) conditions. Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b) showed statistically significant differences ( $p < 0.05$ )

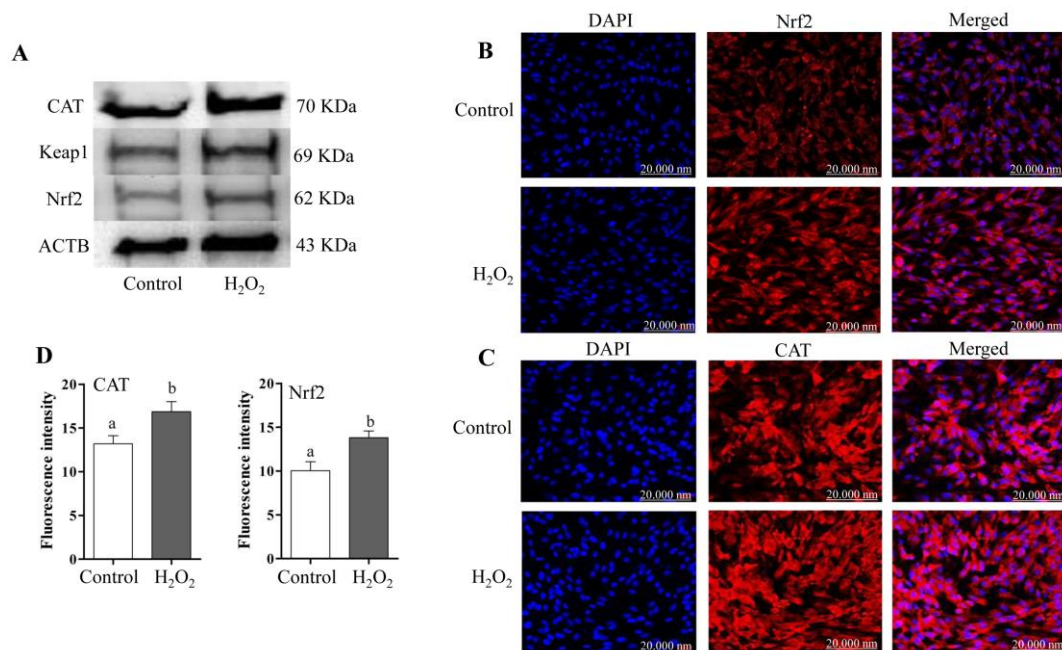


Figure 2.5: Immunoblotting analysis of Nrf2, Keap1 and CAT proteins (A) and immunocytochemistry of Nrf2 (B) and CAT (C) in bovine granulosa cells under oxidative stress and normal conditions while, (D) the fluorescence intensity analysis of Nrf2 and CAT proteins signal. The red colour indicated the expression of proteins, while the blue colour indicated the nuclear staining using 4',6-diamidino-2-phenylindole (DAPI). Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b) showed statistically significant differences ( $p < 0.05$ )

### 2.4.5 Expression of cell proliferation, differentiation, apoptosis marker genes under oxidative stress conditions

Cell proliferation and cell cycle assay results were consistent with the mRNA and protein expression levels of candidate marker genes. Granulosa cells under oxidative stress conditions showed significant reduction in mRNA and protein levels of cell proliferation markers (PCNA and CCDN2) and anti-apoptotic (BCL2L1) marker genes. On the other hand, the mRNA and protein expression levels of genes related to differentiation (CYP11A1 and STAR1) and pro-apoptosis (Casp3) were higher in H<sub>2</sub>O<sub>2</sub>-challenged cells as compared to the control (Figures 6A, B and C).

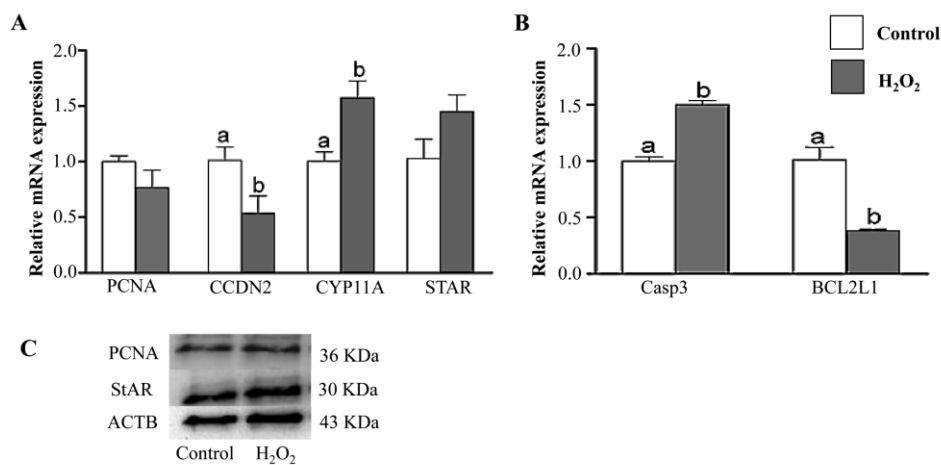


Figure 2.6: Quantitative RT-PCR analysis of cellular proliferation and differentiation marker genes (A) and pro- and anti-apoptotic marker genes (B) in granulosa cells under normal (white bar) or oxidative stress (dark bar) conditions. Western blot analysis of PCNA and StAR1 proteins (C). Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b) showed statistically significant differences ( $p < 0.05$ )

### 2.4.6 Exosomes released into culture media contain mRNA of Nrf2 and antioxidants

The identity of exosomes isolated from culture supernatant was confirmed by western blot analysis of marker proteins (CD63 and Alix). The absence of any detectable protein band for cytochrome c (CYCS) confirmed the purity of exosomes isolated using ultracentrifugation technique (Figure 2.7A). Nanoparticle tracking results revealed that, cells released higher concentration of exosomes with distinct size to culture media when exposed to oxidative stress as illustrated in figure 2.6B. Moreover, the electron microscope results confirmed the size range of the isolated exosomes using the ultracentrifugation technique (Figures 2.7B and C).

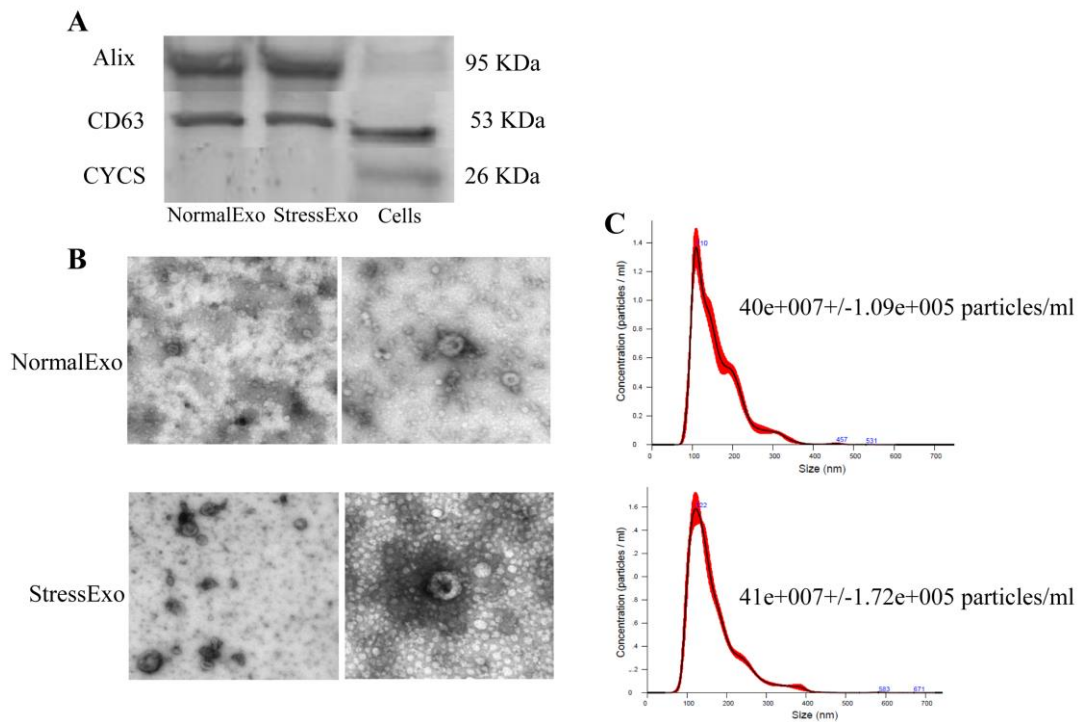


Figure 2.7: Granulosa cells exposed to oxidative stress released high concentration of exosomes with distinct size to extracellular space compared to those cultured under normal conditions. Western blot analysis of exosomes marker proteins (A). Electron microscope analysis (B) and nanoparticles tracking analysis (C) of exosomes released from granulosa cells under normal (NormalExo) and oxidative stress (StressExo) conditions {The Y-axis indicated the concentration (particles/mL) and X-axis indicated the size (nm)}

Exosomes released into culture media from both H<sub>2</sub>O<sub>2</sub>-treated (StressExo) and untreated granulosa cells (NormalExo) were used for RNA isolation and investigation of the abundance of Nrf2 and its downstream antioxidant genes using qRT-PCR. Exosomes released from bovine granulosa cells under oxidative stress conditions were enriched with mRNA encoded by Nrf2 and had lower level Keap1. Moreover, exosomes released under oxidative stress conditions (StressExo) contained mRNA of CAT and TXN1 and significantly lower level of PRDX1 and HMOX1 mRNA compared to those exosomes released by untreated cells (NormalExo). On the other hand, exosomes from both groups did not show any difference in their mRNA content for SOD1 gene. Interestingly, NQO1 mRNA was not detected in exosomes of both treatment groups (Figure 2.8).

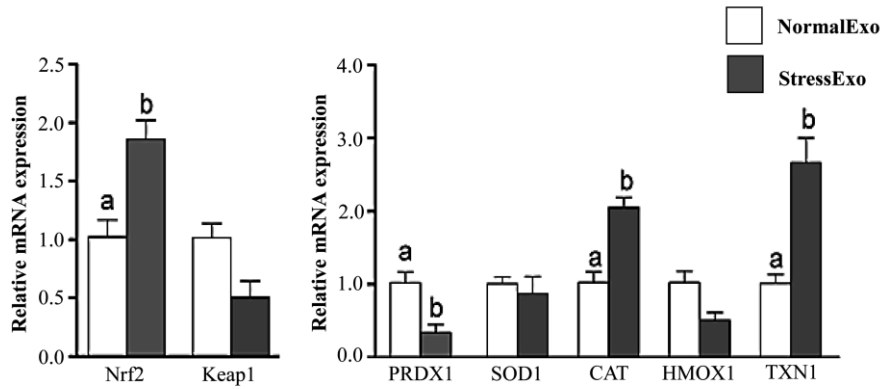


Figure 2.8: Exosomes released from granulosa cells under oxidative stress conditions contained significantly higher mRNA level of Nrf2 and antioxidant genes compared to control group. The relative mRNA expression level of Nrf2 and antioxidant genes in exosomes released from granulosa cells under normal (white bar) and oxidative stress (dark bar) conditions as analysed by qRT-PCR. Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b) showed statistically significant differences ( $p < 0.05$ )

#### 2.4.7 Horizontal transfer of oxidative stress defense mechanism in granulosa cells through exosomes

The uptake of labeled exosomes by granulosa cells during co-culture was confirmed under confocal microscope (Figure 2.9). The co-incubation of granulosa cells with StressExo resulted in lower intracellular ROS level and higher mitochondrial activity (Figures 2.10 and 2.11). Granulosa cells co-cultured under normal conditions with StressExo or NormalExo showed increased cell proliferation (Figure 2.12A). Similarly, cell cycle assay results demonstrated higher proportion of cells arrested at G<sub>0</sub>/G<sub>1</sub> phase

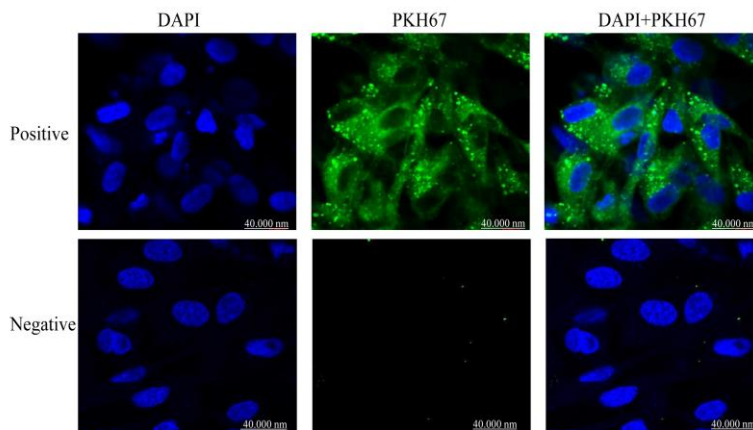


Figure 2.9: Up take of PKH67 labeled exosomes by granulosa cells after co-incubation in vitro. While green colour indicated labeled exosomes, blue colour represented nuclear staining using 4',6-diamidino-2-phenylindole (DAPI). Images were captured under confocal microscope

and a lower proportion in G<sub>2</sub>/M phase in both cells co-cultured with StressExo or NormalExo under oxidative stress condition (Figure 2.12B).

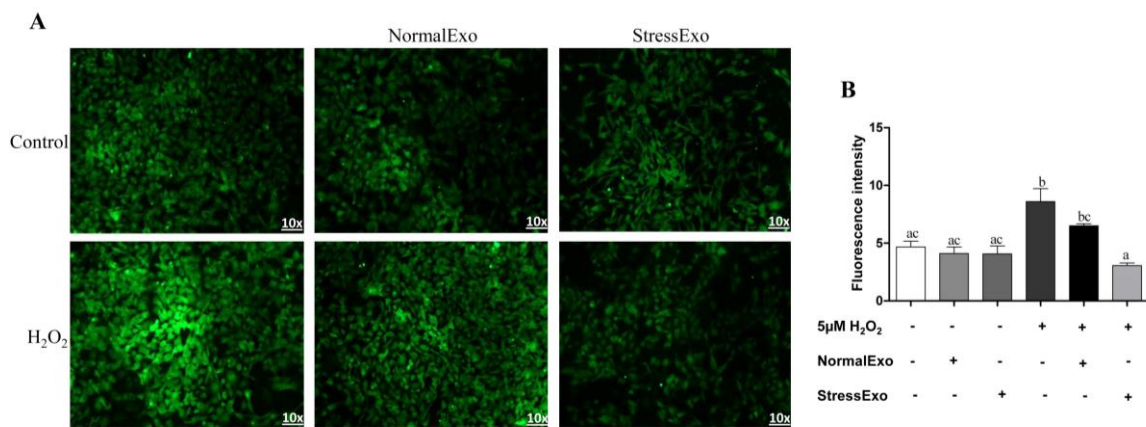


Figure 2.10: Granulosa cells co-incubated with exosomes showed a reduction in ROS level under oxidative stress or normal conditions compared to control ones. The ROS level (A) was measured under normal or oxidative stress conditions in granulosa cells co-incubated with exosomes released under normal (NormalExo) or stress (StressExo) conditions compared to groups cultured without exosomes. The ROS fluorescence intensity analysis (B). Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b, c) showed statistically significant differences ( $p < 0.05$ )

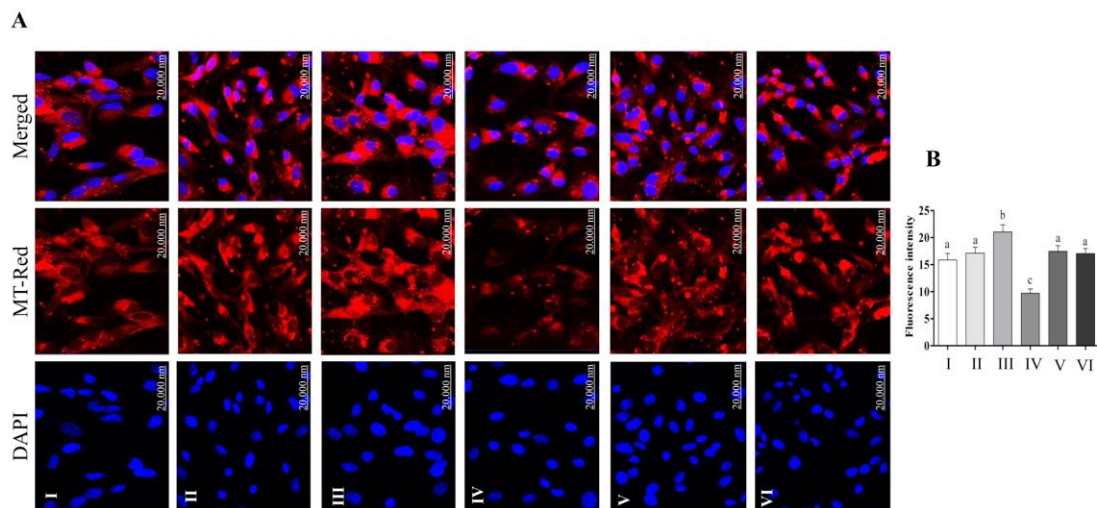


Figure 2.11: The mitochondrial activity (A) and fluorescence intensity analysis (B) in granulosa cells co-incubated with NormalExo (II), StressExo (III) compared to those not co-incubated with any exosomes (I) under normal conditions and the same treatment groups under oxidative stress conditions (IV – VI). The red colour indicated the MT-RED, while the blue colour indicates the nuclear staining using 4',6-diamidino-2-phenylindole (DAPI). Data fluorescent intensity signals (B) are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b, c) showed statistically significant differences ( $p < 0.05$ )



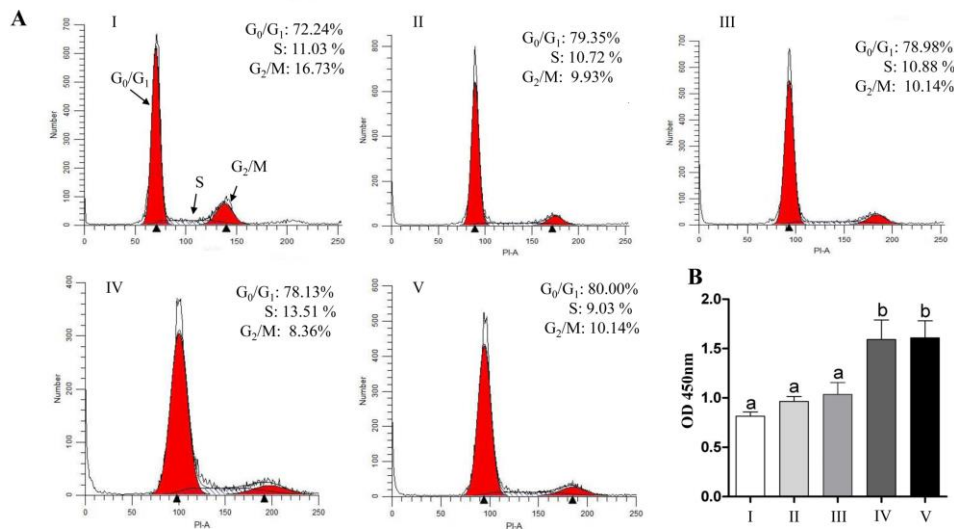


Figure 2.12: Co-incubation of bovine granulosa cells with StressExo altered the cell cycle (A) and cell proliferation (B) profiles under normal or oxidative stress conditions. Granulosa cells co-incubated without exosomes (I), NormalExo (II), StressExo (III) under normal conditions or without exosomes (IV), NormalExo (V), StressExo (VI) under oxidative stress conditions. The Y-axis indicated the cell count and X-axis indicated the DNA content of cells detected by PI staining. Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b) are statistically significant ( $p < 0.05$ )

#### 2.4.8 Co-culture of granulosa cells with StressExo increased the mRNA and protein levels of Nrf2 and antioxidants

To investigate whether co-incubation of granulosa cells with exosomes has resulted in transfer of cargo molecules especially related to oxidative response elements. Bovine granulosa cells were co-cultured with StressExo and NormalExo either under oxidative stress or normal condition. Quantitative RT-PCR results showed that the mRNA expression level of Nrf2 was significantly higher in granulosa cells co-cultured with StressExo compared to those co-cultured with NormalExo in both under oxidative stress and normal conditions. Similarly, downstream genes of Nrf2 (CAT, PRDX1 and TXN1) showed higher abundance in granulosa cells co-cultured with StressExo compared to those cocultured with NormalExo (Figure 2.13). Similar expression was detected for Nrf2 protein level in granulosa cells co-cultured with StressExo. Higher expression of CAT protein was found in granulosa cells co-cultured with StressExo compared to those cultured with NormalExo under normal condition (Figure 2.14).

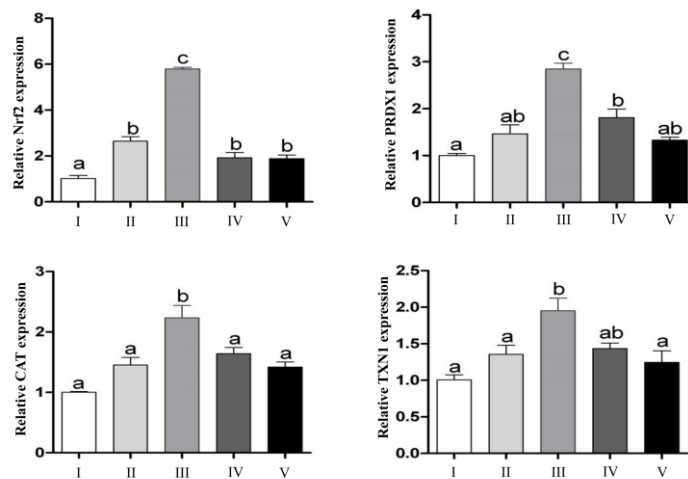


Figure 2.13: Higher mRNA expression level of Nrf2 and antioxidant genes was detected under oxidative stress conditions in granulosa cells co-incubated with StressExo. Expression level of Nrf2 and antioxidant genes in cells cultured under oxidative stress conditions (I), co-incubated with NormalExo (II) or StressExo (III) and cells cultured under normal conditions with NormalExo (IV) or StressExo (V). Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b, c) showed statistically significant differences ( $p < 0.05$ )

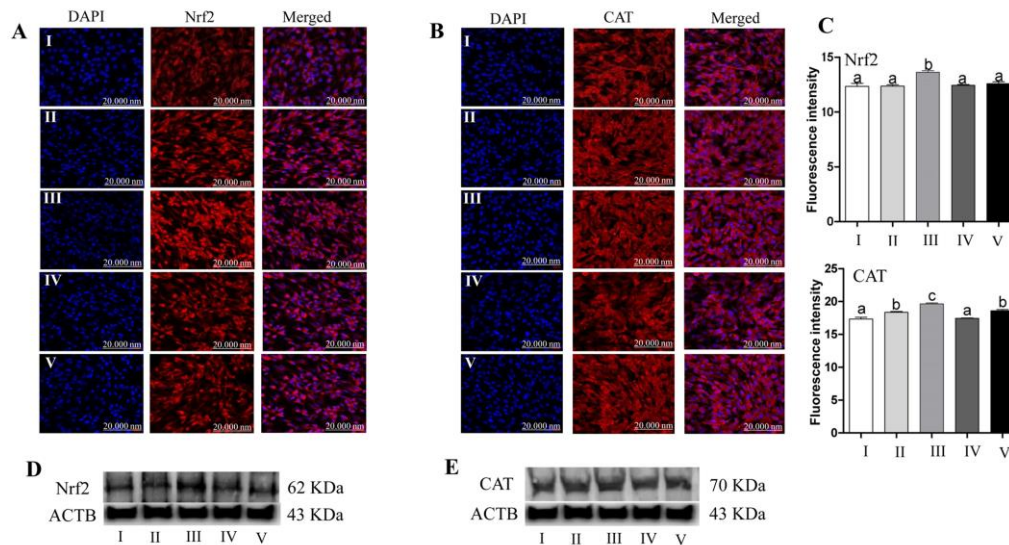


Figure 2.14: The protein expression level of Nrf2 and CAT in granulosa cells co-incubated with exosomes was in the same line with their mRNA expression level. The immunocytochemistry of Nrf2 (A) and CAT (B) in bovine granulosa cells co-incubated without exosome (I), NormalExo (II) or StressExo (III) under oxidative stress conditions or under normal conditions co-incubated with NormalExo (IV) or StressExo (V). The fluorescence intensity analysis (C) of Nrf2 and CAT immunocytochemistry signals. The western blot results of all groups for Nrf2 (D) and CAT (E) proteins. In the immunocytochemistry pictures the red colour indicated the expression of proteins, while the blue colour indicated the nuclear staining using 4',6-diamidino-2-phenylindole (DAPI). Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b, c) are statistically significant ( $p < 0.05$ ).

## 2.5 Discussion

In dairy cattle, oxidative stress can be a result of several environmental and physiological factors such as heat stress, diet, high milk production, negative energy balance, diseases (Bernabucci et al. 2002, Weiss et al. 2004, Bernabucci et al. 2005, Castillo et al. 2006, Guo et al. 2013), that can lead to numerous deleterious effects on female reproduction and fertility (Guerin et al. 2001, Agarwal et al. 2005). Despite the fact that oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidants (Betteridge 2000, Burton and Jauniaux 2011), large set of evidences are available for the critical physiological role of ROS particularly during folliculogenesis and ovulation being locally produced within the follicle by endothelial cells, neutrophils and macrophages (Brannstrom and Norman 1993). However, excess ROS level including  $H_2O_2$  leads to granulosa cells apoptosis and subsequently oocyte dysfunction (Yang et al. 2017). Granulosa cells are layers of somatic cells that are surrounding the oocyte and play a vital role for successful folliculogenesis and subsequently embryo development and pregnancy outcome (Shiota et al. 2003, Assou et al. 2010). Granulosa cells and their antioxidant system during maturation are responsible for preventing oocyte from oxidative stress damage (Tripathi et al. 2013, Adeldust et al. 2015). Excess ROS level in granulosa cells results apoptosis (Tamura et al. 2008, Devine et al. 2012, Tanabe et al. 2015), which in turn resulted in follicular atresia and ovarian dysfunction (Qu et al. 2012). Several evidences are accumulated for the effect of exogenous oxidative stress induced by  $H_2O_2$  resulted in higher ROS accumulation which is harmful for DNA, lipid, protein as well as mitochondria activity and integrity and subsequently lead to granulosa cell apoptosis (Chernyak et al. 2006, Schieber and Chandel 2014, Tanabe et al. 2015, Zhang et al. 2016, Yang et al. 2017). In agreement with this, in the present study exposure of bovine granulosa cells to moderate level of  $H_2O_2$  resulted in higher intracellular ROS level accompanied with lower mitochondrial activity compared to untreated control (Figures 2.1 and 2.2). Even though mitochondria are considered one of the main sources of ROS in mammalian cells (Turrens 2003, Andreyev et al. 2005), their integrity is essential for steroidogenesis in granulosa cells (Rasbach and Schnellmann 2007, Tanabe et al. 2015). Therefore, the reduction in activity of mitochondria in stressed granulosa cells in the present study may result in disturbances in the energy metabolism of those granulosa cells and proper steroidogenesis is impaired.



Cell cycle is a process regulated by growth factors that control different cellular pathways such as proliferation (Berridge 2012). It has been reported that  $H_2O_2$  induced  $G_2/M$  cell cycle arrest and prevented osteoblasts cell proliferation by the reducing the expression of cyclin B1 (Li et al. 2009). Similarly, our results revealed that the proportion of cells under  $G_0/G_1$  phase were reduced while; higher proportion of cells were arrested at  $G_2/M$  phase in  $H_2O_2$ -challenged granulosa cells which is associated with a reduction in cell proliferation rate (Figure 2.3). This was further validated with the expression level of transcripts related to cell proliferation and cell cycle (PCNA and CCDN2). Moreover, significantly higher expression of pro-apoptotic marker gene (Casp3) with concomitant reduction of anti-apoptotic gene (BCL2L1) was observed in granulosa cells under oxidative stress condition as it has been reported before (Yang et al. 2017).

The mammalian cells are outfitted with an assortment of antioxidants that serve to offset the impact of oxidative stress. The harmonic expression of these antioxidant genes eliminates the stress in order to reach the homeostasis state in a manner of prohibiting damage to cellular components that are sensitive to oxidative stress (Bryan et al. 2013). Nrf2-mediated oxidative stress response is one of the most important cytoprotective mechanisms for antioxidant induction and it is sequestered in cytosol by Keap1 protein. Under oxidative stress conditions, Nrf2 is released from Keap1, translocated to nucleus, binds to antioxidant response elements (ARE) and results in releasing of antioxidant molecules (Singh et al. 2010). We have previously shown that the activation of Nrf2 and its downstream antioxidant genes are vital for the survival of bovine embryos under suboptimal culture conditions (Amin et al. 2014, Jin et al. 2016). Similarly, in the present study we have evidenced the induction of the mRNA and protein of Nrf2 and its downstream antioxidants (PRDX1, SOD1, CAT, HMOX1, TXN1 and NQO1) in bovine granulosa cells in response to oxidative stress. Moreover,  $H_2O_2$ -challenged cells showed morphological changes accompanied with semi rounded and shrunken plasma membrane (Figure 2.1B). Previous studies reported that  $H_2O_2$  affects cell membrane permeability by altering changes in membrane composition and stage of cell cycle (Bienert et al. 2006). The exposure of osteosarcoma cells to  $H_2O_2$  resulted in rounded shape and detachment of cells (Wang et al. 2017). These findings may a result of dysregulation of focal adhesion and adhesion skeleton (Song et al. 2010), which may

result in increased distance and gaps between cells and result in impaired cell-to-cell communication.

The communication between various follicular somatic cells and the gamete during folliculogenesis can be either via gap junctions or through signal molecules mediated by the extracellular environment mainly follicular fluid. The presence and potential role of extracellular vesicles especially exosomes in follicular fluid has been reported in mare (da Silveira et al. 2012), women (Santonocito et al. 2014) and cow (Sohel et al. 2013, Navakanitworakul et al. 2016). The quality and quantity of these extracellular vesicles vary depending on the physiological status of the cells, from where they are released (Santonocito et al. 2014, Ailawadi et al. 2015, Hung et al. 2015) and are highly triggered by various stresses factors including diseases, heat stress as well as oxidative stress (Harmati et al. 2017). In agreement with that, our results demonstrated that culture media containing granulosa cells exposed to stress contain higher concentration of exosomes compared to those released by granulosa cells under normal condition (Figure 2.7). Indeed, exosomes released under stress conditions are obviously differed in their RNA and protein contents compared to those released from physiologically normal cells and depending also on the type of stress the cells are exposed to (Eldh et al. 2010, Jong et al. 2012). Therefore, we were aiming at proofing the hypothesis that exosomes released from bovine granulosa cells under stress condition may contain molecules associated with oxidative stress defense mechanism. Accordingly, exosomes released from bovine granulosa cells exposed to oxidative stress contained significant copy of mRNA molecules encoded by Nrf2 and selective antioxidant molecules into extracellular space. As it has been shown in figure 2.4 the enrichment of antioxidant transcripts in exosomes released by the granulosa cells in response to oxidative stress is not valid for all antioxidants. Exosomal mRNA level of Nrf2, CAT and TXN1 was significantly higher in StressExo compared to NormalExo unlike PRDX1 which showed significantly lower mRNA level in StressExo compared to NormalExo. On the other hand, there was no significant difference for mRNA level of SOD1 and HMOX1 which tended to be lower in StressExo compared to NormalExo. Interestingly, NQO1 was not detected at mRNA level in both groups (Figure 2.8). The mechanism of selection and packaging of selective antioxidants into extracellular space through exosomes is so far not clear and it is a subject for future research.

There are several mechanisms for the uptake of exosomes by recipient cells including endocytosis (Feng et al. 2010, Hannafon and Ding 2013), simple fusion (Clayton et al. 2004, Montecalvo et al. 2012) and exosomal surface ligands (Kahlert and Kalluri 2013, Yousefpour and Chilkoti 2014). The uptake of exosomes through either of these mechanisms resulting in functional alterations in recipient cells depending on the cargo molecule they are carrying with (Sohel et al. 2013, Braccioli et al. 2014). In the present study, we were aiming at validating the potential horizontal transfer of exosome mediated oxidative stress defense molecules among bovine granulosa cells. Following co-incubation of StressExo with cultured granulosa cells there was a significant increase in cellular mRNA levels of Nrf2 and its selective downstream antioxidants (CAT, PRDX1 and TXN1) (Figure 2.13). Similarly, a significant increase in protein expression of NRF2 and CAT genes was observed in granulosa cells co-incubated with StressExo. Catalase (CAT) is an enzyme that can deactivate one million free radicals per second per molecule in a single cycle of catalytic reaction (Haney et al. 2015). We have evidenced in the first experiment that alteration in relative abundance of cellular defense molecules was also accompanied by a reduction in ROS accumulation and the corresponding increase in mitochondrial activity under oxidative stress conditions (Figures 2.1 and 2.2). Similarly, due to the horizontal transfer of oxidative stress defense molecules through exosomes, granulosa cells co-cultured with StressExo showed a reduced ROS accumulation and the corresponding improved mitochondrial activity (Figures 2.10 and 2.11). Moreover, we have also evidenced that co-incubation of bovine granulosa cells with StressExo resulted in an increase in the proportion of cells under  $G_0/G_1$  phase and decrease in proportion of cells at  $G_2/M$  phase, which is associated with increased proliferation rate under oxidative stress condition (Figure 2.12). Taken together, these results suggest that oxidative stress-released exosomes carry antioxidant molecules defense molecules which can be uptaken by the neighboring cells to enrich their cellular defense mechanism in order to protect themselves against oxidative stress condition induced by the unfavorable environment.

## 2.6 Conclusion

The present study provides evidence that the survival of granulosa cells under oxidative stress condition is dependent on their ability to activate their Nrf2 mediated oxidative stress response mechanisms. Moreover, these several cellular cascades of antioxidant

molecules can also be released into extracellular space being coupled with exosomes which have a great potential in transfer of defense molecules from one cells to the others.

## 2.7 Abbreviations

DAPI: Complexes 4',6-diamidino-2-phenylindole, DMEM/F-12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide, PBS-CMF: Ca<sup>2+</sup>/Mg<sup>2+</sup> free 1x phosphate buffer saline, ROS: Reactive oxygen species, NormalExo: Exosomes released from granulosa cells under normal conditions, StressExo: Exosomes released from granulosa cells under oxidative stress conditions, mRNA: Messenger RNA, qRT-PCR: Quantitative real time PCR, ACTB: Actin beta, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, 18S: 18s ribosomal RNA, Nrf2: Nuclear factor, erythroid 2 like 2, Keap1: Kelch like ECH associated protein 1, CAT: Catalase, PRDX1: Peroxiredoxin 1, SOD1: Superoxide dismutase 1, HMOX1: Heme oxygenase 1, TXN1: Thioredoxin, NQO1: NAD(P)H quinone dehydrogenase 1, PCNA: Proliferating cell nuclear antigen, STAR1: Steroidogenic acute regulatory protein, Casp3: Caspase 3, apoptosis-related cysteine peptidase, BCL2L1: BCL2 like 1, CD63: CD63 molecule, Alix: ALG-2 interacting protein X, CYCS: Cytochrome c

## 2.8 Declarations

### Ethics approval and consent to participate

The study was conducted on bovine granulosa cells derived from ovaries collected from local abattoir and thus special approval of this experiment was not essential.

### Consent for publication

Not applicable.

### Availability of data and materials

All data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

This experiment received no specific funding.

### Authors' contributions

MSZ designed the experiment, performed all laboratory experiments involving mRNA, protein, phenotypes analysis and exosome related works, analysed the data, draft the manuscript; LL participated in cell culture and gene expression analysis; DSW participated in organization and analysis of the data. EH, CN read the manuscript and commented on the experimental design. ET participated in data analysis. MH participated in interpreting the results, reading the manuscript. KS participated in the design of the experiment, supervision of the experiment. DT designed the experiment, guided the study, involved in interpreting the results and reviewing the manuscript.

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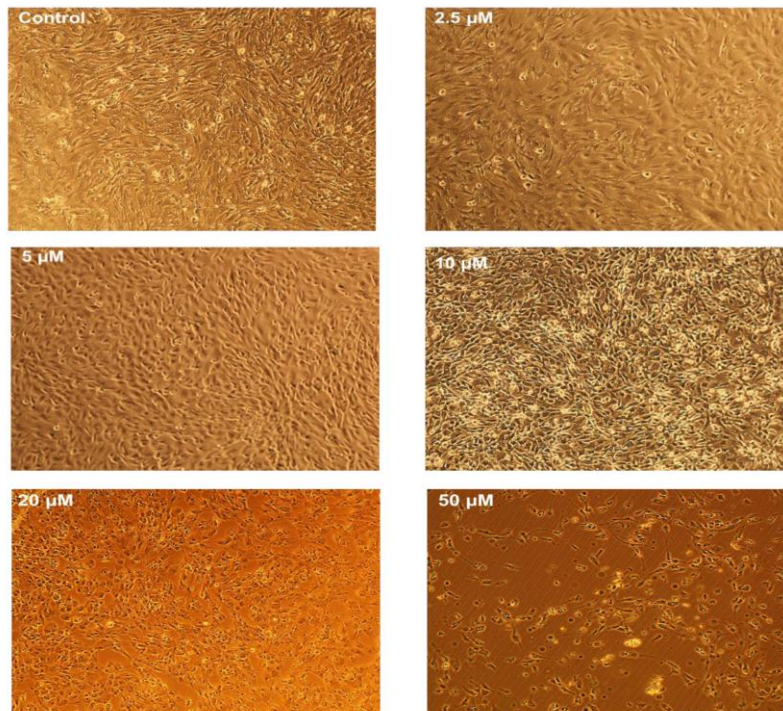


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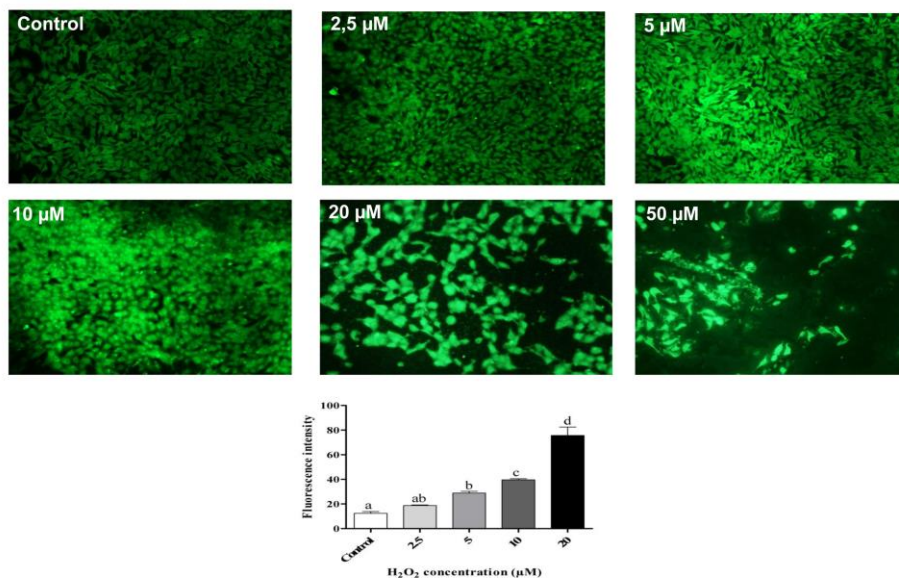
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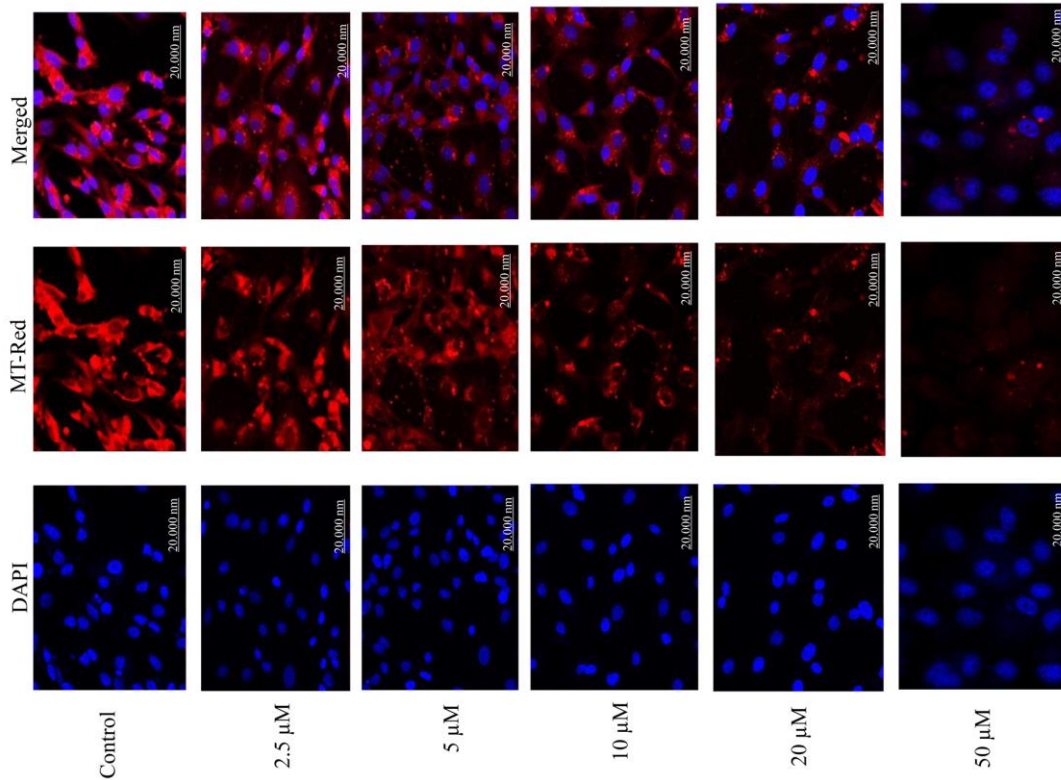
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2.10 supplementary data related to H<sub>2</sub>O<sub>2</sub> optimization

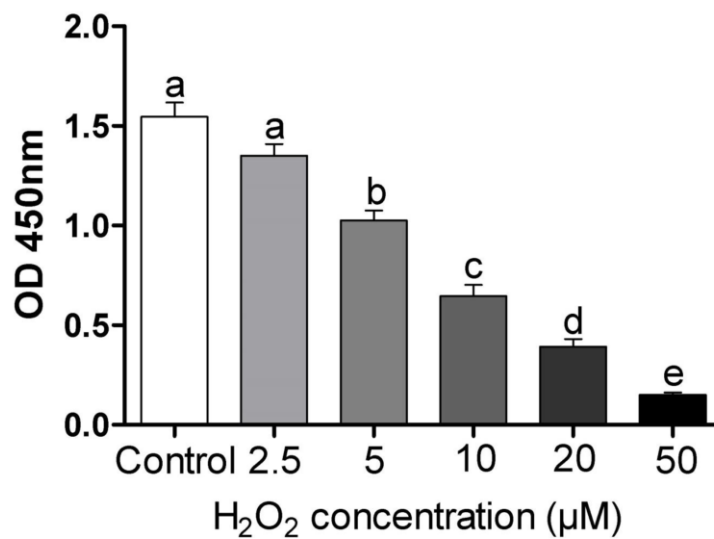
Supplemented figure 2.1: H<sub>2</sub>O<sub>2</sub> altered bovine granulosa cell morphological changes and induced cell death in dose dependent manner



Supplemented figure 2.2: H<sub>2</sub>O<sub>2</sub> induced ROS accumulation in bovine granulosa cells treated with H<sub>2</sub>O<sub>2</sub> in dose dependent manner. Data of ROS fluorescence intensity analysis are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b, c, d) showed statistically significant differences ( $p < 0.05$ )



Supplemented figure 2.3: High intracellular ROS level was accompanied with low mitochondrial activity in bovine granulosa cells treated with different concentrations of  $\text{H}_2\text{O}_2$



Supplemented figure 2.4. Oxidative stress induced by different concentration of  $\text{H}_2\text{O}_2$  resulted in reduced granulosa cell proliferation. Data are mean  $\pm$  SEM from four independent biological replicates. Bars with different letters (a, b, c, d, e) are statistically significant ( $p < 0.05$ )

Chapter 3: DNA methylation mediated regulation of focal adhesion pathway  
(Manuscript submitted to BMC Genomics: under revision)

**Hyaluronic acid and epidermal growth factor improved the bovine embryo quality by regulating the DNA methylation and expression patterns of the focal adhesion pathway**

Mohammed Saeed-Zidane<sup>1,4</sup>, Dawit Tesfaye<sup>1,2, 3</sup>, Yousri Mohammed Shaker<sup>4</sup>, Ernst Tholen<sup>1</sup>, Christiane Neuhoff<sup>1</sup>, Franca Rings<sup>2</sup>, Eva Held<sup>1,2</sup>, Michael Hoelker<sup>1,2,3</sup>, Karl Schellander<sup>1,2,3</sup>, Dessie Salilew-Wondim<sup>1</sup>

<sup>1</sup>Institute of Animal Science, Department of Animal Breeding and Husbandry, University of Bonn, Bonn, Germany

<sup>2</sup>Teaching and Research Station Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany

<sup>3</sup>Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

<sup>4</sup>Animal and Poultry Physiology Department, Animal and Poultry Production Division, Desert Research Center, Mataria, Cairo, Egypt

\*Correspondence author:

Dessie Salilew-Wondim, PhD

Institute of Animal Science, University of Bonn

Endenicher Allee 15, 53115 Bonn, Germany

Email: dsal@itw.uni-bonn.de

Phone: 0049-228/73-3586

Fax: 0049-00228/73-2284



### 3.1 Abstract

#### Background

The focal adhesion (FA) pathway is one of the key molecular pathways affected by suboptimal culture conditions during embryonic development. The epidermal growth factor (EGF) and hyaluronic acid (HA) are believed to be involved in the FA pathway by regulating the adherence of the molecules to the extracellular matrix. However, how the EGF and HA could influence the embryo development via the focal adhesion pathway is not clear. Therefore, this study was aimed to investigate the effect of continued or stage specific supplementation of EGF and/or HA on the developmental competence and quality of bovine preimplantation embryos and the subsequent consequences on the expression and DNA methylation patterns of the focal adhesion pathway genes.

#### Results

Supplementation of EGF, HA or EGF + HA from zygote to the blastocysts stage reduced the level of reactive oxygen species, decreased the apoptotic cells and increased the expansion and hatching rate of the blastocysts after thawing. Gene expression and DNA methylation analysis in the resulting blastocysts indicated that combined supplementation of EGF and HA increased the expression of genes involved in the FA pathway while supplementation of EGF, HA or a combination of EGF and HA (EGF + HA) during the entire preimplantation period changed the DNA methylation patterns of the genes involved in focal adhesion pathway. On the other hand, supplementation of culture media with EGF + HA until the 16-cell stage relatively increased the day 7 blastocysts rate which exhibited higher expression level of genes involved in the focal adhesion pathway compared to those supplemented after the 16-cell stage. Conversely, the DNA methylation level of candidate genes was increased in the blastocysts obtained from embryos cultured in media supplemented with EGF + HA after 16-cell stage.

#### Conclusion

Supplementation of bovine embryos with EGF and/or HA during the entire preimplantation period or in a stage specific manner modified the DNA methylation and expression patterns of candidate genes involved in the FA pathway which was in turn associated with the observed embryonic developmental competence and quality.

Key words: DNA methylation, Gene expression, Bovine, Embryo, culture condition

### 3.2 Background

Suboptimal culture conditions during the preimplantation period can result in long-term effects on embryo competence and pregnancy establishment. Despite many attempts to modulate the *in vitro* culture conditions to mimic the *in vivo* environment, the quality of *in vitro* produced embryos remains low (Lonergan et al. 2003). Suboptimal embryo culture condition decreases the quality and hinders the developmental competence of the embryo by altering the expression and DNA methylation patterns of developmentally related genes and pathways (Gad et al. 2012, Salilew-Wondim et al. 2015). Among these, the focal adhesion pathway is one of the top dysregulated pathways in bovine embryos due to suboptimal culture conditions. Focal adhesion is one of the cell communication mechanisms which are vital for cell motility, differentiation, migration, proliferation and cell survival (Kaneko et al. 2012, Wrighton 2013). Many of the focal adhesion proteins, such as  $\beta 1$  integrin,  $\alpha 4$  integrin,  $\alpha 5$  integrin, talin, paxillin, vinculin, focal adhesion kinase and integrin-like kinase are essential for embryonic development. Functional loss of these proteins during embryogenesis would affect the cell-extracellular matrix (ECM) adhesion, cytoskeletal organization, polarity, migration and survivability of the embryos (Bladt et al. 2003).

Adhesion to the ECM with the supplementation of growth factors is necessary for normal cell growth (Renshaw et al. 1999) and supplementation of growth factors in cell-free culture media was found to improve the blastocyst rate (Sinclair et al. 2003). Among these factors, epidermal growth factor (EGF) was found to improve the embryonic development in mouse (Adamson 1993), pig (Wei et al. 2001) and bovine (Cebrian-Serrano et al. 2014). Similarly, hyaluronic acid (HA), which is one of the main components of ECM, is also believed to improve the blastocyst rate of *in vitro* produced bovine embryos (Block et al. 2009). However, the molecular mechanism how supplementation of EGF and HA could influence the embryonic development via focal adhesion pathway is not clear. Here, we hypothesized that the growth factors and the extracellular components could improve the quality and development of embryos by regulating DNA methylation and expression patterns of genes involved in the focal adhesion pathway. Indeed, suboptimal *in vitro* culture conditions could cause DNA methylation changes during embryo development (Katari et al. 2009, Reis e Silva et al. 2012). The dynamic changes in the DNA methylation pattern of embryos during *in vitro* development may rely on epigenetic adaptability of embryos resulting from the cellular

interactions with extracellular environment via cell adhesion to ECM molecules. Therefore, this study was aimed to investigate the effect of epidermal growth factor (EGF) and/or hyaluronic acid (HA) supplementation on the developmental competence and quality of the bovine preimplantation embryos and its subsequent significance on the expression and DNA methylation patterns of the genes involved in focal adhesion pathway.

### 3.3 Materials and methods

#### 3.3.1 Experimental design

Two consecutive experiments were conducted to achieve the goals of the study. In the first experiment, supplementation of EGF and/or HA was performed from zygote to the blastocysts stage (“the entire preimplantation period”) For this, synthetic oviduct fluid (SOF) culture media supplemented with fatty acid free bovine serum albumin was considered as the basic media (BM). Therefore, in the first experiment (Figure 3.1A), *in vitro* produced presumptive zygotes were cultured only in BM (control group), or BM supplemented with 10 ng/mL EGF, BM supplemented with 1 mg/mL HA or BM supplemented with a combination of 10 ng/mL EGF and 1 mg/mL HA (EGF + HA). Afterwards, the effect of EGF and/or HA on the developmental competence and quality of the preimplantation embryo was recorded. The mRNA expression and DNA methylation pattern of genes involved in focal adhesion pathway was investigated in the resulting blastocyst. In the second experiment, preimplantation embryos were supplemented with a combination of EGF and HA in a stage specific manner that coincided with pre and post embryonic genome activation (Figure 3.1B). Accordingly, preimplantation embryos were then cultured in six culture conditions and six blastocyst groups were generated for development assessment and molecular analysis. The first blastocyst group was generated from zygotes cultured in basic media (BM), while the second blastocyst group (After\_4C) was generated from zygotes culture in BM until 4-cell and then supplemented with 10 ng/mL EGF and 1 mg/mL HA (EGF + HA) until blastocyst stage. The third blastocyst group (After\_16C) was derived from zygotes cultured until 16-cell stage in BM followed by EGF + HA supplementation until the blastocyst stage while the fourth blastocyst group (All-stage) was obtained from zygotes cultured until blastocyst stage in BM supplemented with EGF + HA. The fifth group (Until\_4C) was obtained from zygotes cultured in BM supplemented with EGF + HA

until 4-cell stage and further cultured until the blastocyst stage in BM, and the sixth blastocyst group (Until\_16C) was generated from zygotes cultured in BM supplemented with EGF + HA until the 16-cell stage and then cultured in BM until blastocyst stage.

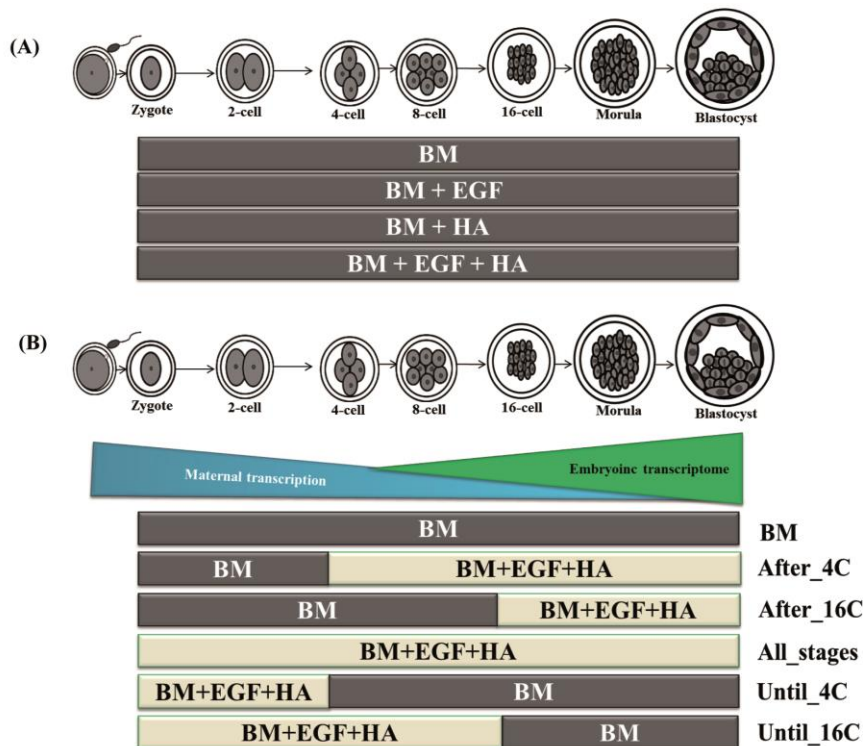


Figure 3.1. Outline of the experimental design. In first experiment (A), zygotes were cultured until the blastocysts stage: only in basic media (BM), BM supplemented with EGF, BM supplemented with HA or BM supplemented with EGF + HA. In the second experiment (B), preimplantation embryos were cultured in BM until the blastocyst stage (BM), or supplemented with EGF + HA after 4 cell stage (After\_4C), after 16 cell stage (After\_16C), until 4-cell (Until\_4C), until 16-cell stage (Until\_16C) or from zygote to the blastocysts stage (All-stage)

### 3.3.2 Oocytes collection and in vitro maturation

Bovine ovaries were collected from local slaughterhouse and transported to the laboratory within 1-2 h in a thermal container containing physiological saline (0.9% NaCl) solution at 37 °C. After arrival, the ovaries were washed with saline solution and rinsed in 70% ethanol. Cumulus oocyte complexes (COCs) were then aspirated from follicles 2-8 mm in size using a 5-mL syringe loaded with an 18- gauge needle. The COCs with more than three compacted cumulus layers and evenly granulated cytoplasm were cultured in groups of 50 in 400 µL of standard maturation medium (tissue culture media (TCM-199) (M-2154; Sigma, Munich, Germany) with Earle salts buffered with 4.43 mM HEPES (H-9136; Sigma, Munich, Germany), 33.9 mM sodium bicarbonate

(S-5761; Sigma, Munich, Germany), 12% estrous cow serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg/mL gentamycin sulphate and 10 µg/mL FSH (Folltropin, Vetrepharm, Canada) in four well dishes (Nunc, Roskilde, Denmark). Oocyte maturation was performed for 22 h at 38.7 °C and 5% CO<sub>2</sub> in air with maximum humidity. Oocyte maturation was determined by the presence of first polar body and cumulus cell expansion.

### 3.3.3 In vitro fertilization and embryo production

A group of 50 matured COCs were in vitro fertilized with  $2 \times 10^6$  spermatozoa/mL in Nunclon dishes in 250 µL modified Tyrode medium supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL bovine serum albumin (BSA), 1 µg/mL heparin, 10 µM hypotaurine, 20 µM penicillamine and 2 µM epinephrine at 38.7 °C. The COCs and sperm were incubated at 5% CO<sub>2</sub> in air with maximum humidity. Eighteen hours later, the presumptive zygotes were cultured in 400 µL of SOF culture medium supplemented with or without EGF and/or HA according to the experimental design indicated above (Figure 3.1) in four well dishes (Nunc, Roskilde, Denmark) covered with mineral oil at 38.7 °C and 5% CO<sub>2</sub> in humidified air. The cleavage and blastocyst rates, blastocysts cell number, apoptotic index, level of reactive oxygen species (ROS) and cryotolerance ability of the embryos were recorded in each treatment group of the first experiment. Moreover, in the second experiment, the cleavage rate and day 7, day 8 and day 9 blastocyst rates were recorded and the blastocysts stage embryos in both experiments were used for gene expression and DNA methylation analysis.

### 3.3.4 ROS accumulation assay

Intracellular ROS level in the blastocyst stage embryos was determined using the cell-permeant 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Life Technologies, USA) following the manufacturer's instruction. Briefly, the blastocysts from each experimental group were incubated in 400 µL media containing µM H2DCFDA for 20 min at 37 °C followed by washing twice with PBS-PVA 0.01%. Thereafter, the ROS level was evaluated under inverted fluorescence microscope (Leica DM IRB, Wetzlar, Germany) and the images were captured using the green fluorescence filter. Afterwards, the image signals were quantified using the Image J 1.48v software.

### 3.3.5 Blastocyst cell number assessment

The total cell number in the blastocysts was determined using Hoechst 33342 stain (Sigma, Munich; Germany). Blastocysts from each group were incubated for 5 min in a solution containing 2% formalin and 0.25% glutaraldehyde. Fixed blastocysts were then mounted and stained with 12.5 µg/mL solution of glycerol-based Hoechst 33342 on clean glass slides for 10 min. Stained nuclei were visualized using the epifluorescent microscope (Olympus, Tokyo, Japan). Finally, the numbers of cells in each blastocyst were counted in each individual blastocyst from each group.

### 3.3.6 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) assay

TUNEL assay kit (Roche<sup>®</sup>, Mannheim, Germany) was used to detect dead cells in blastocysts of different treatment group. Blastocysts were fixed, permeabilized and then incubated with drops of TUNEL solutions for 1 h in a humid chamber at 37 °C in dark. Samples used as positive control were treated with DNase (Promega, WI; USA) and the negative controls were incubated only with making solution. Thereafter, samples were washed and stained with Hoechst 33342 (Sigma, Munich; Germany), then mounted with glycerol on clean slides. The samples were observed under the fluorescence microscope (Leica, Germany) and TUNEL positive (fragmented DNA) cells were recorded.

### 3.3.7 Cryotolerance test

To investigate the effect of EGF and/or HA on embryo freezability, the blastocyst stage embryos were subjected to the cryotolerance test. Briefly, the day 7 blastocysts were washed with D-PBS containing 5% PVA and then transferred to the solution consisting of 1.5 M ethylene glycol and 0.1 M sucrose (BoviFreeze, Minitube GmbH, Tiefenbach, Germany). Groups of 10-15 blastocysts were loaded into an open-pulled straw (Minitube GmbH, Tiefenbach, Germany) and then immediately plunged into the programmable freezer “Freeze Control” (Consarctic, Germany). Seeding was accomplished manually with forceps cooled in liquid nitrogen at a temperature of -6 °C, followed by a cooling to -30 °C at a rate -0.3 °C /min. Thereafter, the straws were kept in liquid nitrogen. One week later, the embryos were thawed and washed with SOF media supplemented with fatty acid free BSA. Finally, the embryos were cultured in 400 µL culture media in four well dishes (Nunc, Roskilde, Denmark) covered with

mineral oil at 38.7 °C in 5% CO<sub>2</sub> and humidified air. The expansion and hatching rates were determined at 24, 32, 48 and 56 h post thawing.

### 3.3.8 DNA and total RNA isolation and first strand cDNA synthesis

The genomic DNA (gDNA) and total RNA from each experimental group were isolated from four biological replicates of day 7 blastocysts (10 blastocysts in each replicate) using RNA/DNA/protein purification plus micro kit (Norgen biotek, Canada) according to manufacturer's instructions. The gDNA was used for DNA methylation analysis whereas the total RNA was used for the gene expression studies. Total RNA concentration was measured using Nanodrop and adjusted RNA concentration was reverse transcribed into cDNA using thermo scientific first strand cDNA synthesis kit (Life technologies, Germany). Briefly, total RNA of each sample was incubated with 0.5 µL oligo-dT and 0.5 µL random primers at 65 °C for 5 min followed by chilling on ice. Afterwards, 1 µL ribolock RNase inhibitor, 4 µL 5x reaction buffer, 2 µL of 10 mM dNTPs mix and 2 µL reverse transcriptase were added and incubated at 25 °C for 5 min, 37 °C for 60 min followed by 70 °C for 5 min. Samples were then stored at -20 °C until use.

### 3.3.9 Gene expression analysis

To determine the effect of EGF and/or HA supplementation during in vitro embryo culture on the expression of genes involved in focal adhesion pathway, the mRNA levels of candidate genes was determined using quantitative real time PCR (qPCR). For that, the mRNA expression level of 11 genes namely, actin gamma1 (ACTG1), focal adhesion kinase (FAK), phosphatase and tensin homolog (PTEN), vinculin (VCL), p21 protein (Cdc42/Rac)-activated kinase 4 (PAK4), ras-related C3 botulinum toxin substrate 1 (RAC1), phosphatase and tensin homolog, collagen, type IV, alpha 1 (COL4A1), collagen, type I, alpha 2 (COL1A2), epidermal growth factor receptor (EGFR), CD44 molecule (CD44) and hyaluronan-mediated motility receptor (HMMR) were selected for analysis. In addition, the expression level of DNA (cytosine-5-)-methyltransferase 1 (DNMT1), DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A) and DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B) were also investigated. For all samples, the qRT-PCR was performed using Applied Biosystem<sup>®</sup> StepOne Plus<sup>™</sup> System (Thermo Fisher Scientific Inc, USA) using iTaq<sup>™</sup> Universal SYBR<sup>®</sup> Green Supermix (Bio-Rad Laboratories GmbH, Germany) with the following

program; 95 °C for 3 min, 40 cycles at 95 °C for 15 sec, 60 °C for 45 min. The data were analyzed using a comparative threshold cycle method ( $2^{-\Delta\Delta CT}$ ) using the beta actin ( $\beta$ -actin) and glyceraldehyde phosphate dehydrogenase (GAPDH) expression as the normalizer. All primers (Table 3.1) used for qRT-PCR were designed using the online software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Table 3.1. List of primers used for quantitative real time PCR analysis

Accession number	Genes	Primer sequence	Size
NM_173979	ACTB	F: 5'-GGCATTACGAACTACCTT-3' R: 5'-CAATCCACACGGAGTACTTG-3'	208
NM_001034034	GAPDH	F: 5'-ACCCAGAAGACTGTGGATGG-3' R: 5'-ACGCCTGCTTACCACCTT-3'	247
NM_001033618	ACTG1	F: 5'-ATCCGAAAGGACCTGTATGC-3' R: 5'-CTTGCTGATCCACATCTGCT-3'	213
NM_001075250	FAK	F 5'-ACTGGATTCAAGGCCA-3' R 5'-CAGCCCTTGTCTGTGAGGTA-3'	231
XM_613125	PTEN	F:5'-TGGGGAAGTAAGGACCAGAG-3' R: 5'-ATTGCAAGTTCCACCACTGA-3'	172
NM_001191370	VCL	F:5'-TTGCAAAGTGTGACCGAGTG-3' R: 5'-CAACAGCTTGATGGGAGTCG-3'	203
NM_001076184	PAK4	F: 5'-TACCAGCATGAGAACGTGGT-3' R: 5'-CTCTTGATGTCCCGGTGGAT-3'	212
NM_174163	RAC1	F: 5'-ACGGAGCTGTGGGTAAGACT-3' R: 5'-TACATCTGTCTGCGGGTAGG-3'	200
NM_001166511	COL4A1	F: 5'-TCTGGATCGGCTACTCCTTT-3' R: 5'-AACATCTCGCTCCGCTCTAT-3'	196
NM_174520	COL1A2	F: 5'-TGAAAAGGTCATGCTGGTC-3' R: 5'-TACCCCTTTCTCCTGGTTTG-3'	200
HM749883	EGFR	F: 5'-GACCCGAAAGAACTGGACAT-3' R: 5'-TGTTATATCCAGGCCGACAA-3'	177
NM_174013	CD44	F: 5'-CTGAAATGAGGGCCAGTTA-3' R: 5'-CCAACCCCACTTGAAAGAAA-3'	285
NM_001206621	HMMR	F: 5'-TGCTTATACTCAGGCCACCC-3' R: 5'-CGGACATCCTCTGCACTTTG-3'	189
NM_182651	DNMT1	F: 5'-TGACTACATCAAGGGCAGCA-3' R: 5'-AGGTTGATGTCTGCGTGGTA-3'	192
NM_001206502	DNMT3B	F: 5'-CTGCTGAATTACACTCGCCC-3' R: 5'-CCAGAAGTATCGGGCTCTGT-3'	177
NM_181813	DNMT3A	F: 5'-AGCACAACGGAGAAGCCTAA-3' R: 5'-CAGCAGATGGTGCAGTAGGA-3'	245



### 3.3.10 Immunoblotting

Western blot was performed to detect the expression levels of the focal adhesion pathway marker proteins (FAK and VCL) in embryos derived from zygotes cultured in media supplemented with or without EGF and/or HA. For this, 30 blastocysts from each treatment group were boiled with 4  $\mu$ L 2x SDS loading buffer at 95 °C for 5 min and loaded on 4-18% gradient SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membrane (Protran®, Schleicher & Schuell Bioscience) and then blocked with Roti-block solution (Carl Roth, Germany) for 1 h at room temperature. The membrane was overnight incubated at 4 °C with anti FAK goat polyclonal antibody diluted at 1:250 (Santa Cruz Biotechnology Inc, Germany). On the second day, the membrane was washed with Tween-Tris-buffer saline (TTBS) and incubated with donkey anti goat secondary antibody diluted at 1:5000 (Santa Cruz Biotechnology Inc, Germany) for 1 h at room temperature. Thereafter, the membrane was washed with TTBS and incubated with peroxide solution and luminal enhancer for 5 min at room temperature in the dark. Images were developed on the ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany) and the detected bands were quantified using the Image J 1.48v software. Afterwards, the membrane was subjected to stripping protocol (Bio-Rad, Germany), followed by blocking with the Roti-block solution (Carl Roth GmbH). The membrane was incubated with anti VCL rabbit polyclonal antibody (1:250) or anti ACTB mouse monoclonal antibody (1:500) (Santa Cruz Biotechnology Inc, Germany).

### 3.3.11 Immunohistochemistry

The localization of VCL protein was performed using an immunohistochemistry assay. Briefly, fifteen blastocysts from each experimental group were washed three times in phosphate- buffer saline (PBS), and then fixed overnight at 4 °C in 4% paraformaldehyde in PBS. Fixed samples were washed twice with glycine in PBS and then permeabilized with 0.5% (v/v) Triton-X100 (Sigma, Munich, Germany) in PBS for 4 h at room temperature. The permeabilized blastocysts were incubated in 3% normal donkey serum (Sigma, Munich, Germany) in PBS for 1 h at room temperature. Samples were incubated overnight at 4 °C with anti VCL rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Germany). Afterwards, blastocysts were incubated at 37 °C for 2 h in the dark with FITC-conjugated goat anti-rabbit secondary antibody (Lifespan

Biosciences, Seattle, WA). Blastocysts were mounted in mounting medium containing DAPI on a clean slid glass and were then visualized under the CLSM LSM-780 confocal laser scanning microscope (Zeiss, Germany). The images were analysed with ZEN 2011 software.

### 3.3.12 Bisulfite sequencing

The genomic DNA (gDNA) isolated from blastocysts of different groups was subjected to bisulfite treatment using EZ DNA methylation direct kit (Zymo Research, USA) and amplified using gene specific primers (Table 3.2). The primers for bisulfite sequencing were designed using the online software (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>). The PCR amplification was performed using 2  $\mu$ L of bisulfite treated gDNA, 1  $\mu$ L of forward primer, 1  $\mu$ L of reverse primer, 12.5  $\mu$ L of 2x reaction buffer, 0.3  $\mu$ L of 10 mM dNTP mix, 0.2  $\mu$ L of Zymo Taq<sup>TM</sup> DNA polymerase (Zymo Research, USA) and nuclease free water was added up to 25  $\mu$ L. The presence of the PCR product was confirmed after loading 5  $\mu$ L of the PCR product on 2% agarose gel electrophoreses run in 1% TE buffer. The remaining PCR product was purified using the QIAquick PCR purification (Qiagen, Germany), cloned to pGEM<sup>®</sup>-T Easy Vector Systems (Promega, WI, USA) and transformed to *E. coli* competent cells. The bacterial culture was then plated onto the LB agar/ampicillin/IPTG/X-gal plate and incubated overnight at 37 °C. Independent clear white colonies were selected for sequencing. For that, the colonies were lysed at 95 °C for 15 min and 10  $\mu$ L of the lysate were used for PCR amplification using M13 primer. The PCR product was then sequenced using the GenomeLab<sup>TM</sup> GeXP Genetic Analysis System (Beckman Coulter). The bisulfite sequencing DNA methylation analysis software (BISMA) was used to analyze the sequencing data (<http://services.ibc.uni-stuttgart.de/BDPC/BISMA/>).

Table 3.2. List of primers used for DNA methylation analysis

Accession number	Genes	Primer sequence	Number of CpGs in PCR product
ENSBTAG00000013472	COL1A2	F: 5'-GGGATTTTAAGTTTATTTTAAATAAA-3'	16
		R: 5'-AACCTACCTACCTTATACCCTAC-3'	
		F: 5'-TGGTTTGGTTGGTAAAGTTTATTTTTT-3'	22
		R: 5'-CCAACCCTACCTACCTTATACCCTAC-3'	
ENSBTAG00000012849	COL4A1	F: 5'GGGTAGGATTTTATATTAGTTTTTGATGTT-3'	7
		R 5'-ACCTTCTCTTAAATTCCCCTTCAAT-3'	
		F: 5'-GATAGAGGAGAAGGGGAATTTAGGAT-3'	9
		R: 5'-AACTCCACCAAAAACCCTATTT-3'	
ENSBTAG00000009233	RAC1	F: 5'-AAGTGAATGGTAGTTTTTAGGAATTT-3'	8
		R: 5'-AAAAAATTATTTACCTCCCATTAATA-3'	
		F: 5'-TTTTTGGGGTTTGGATATTTGTAT-3'	14
		R: 5'-AAACCAAACACCCCTAAAACATAAAC-3'	

### 3.3.13 Statistical analysis

The data of this study were analyzed using Statistical Analysis System (SAS) version 9.1 software (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) followed by Tukey multiple pairwise comparison was performed between treatment groups. Number of blastocysts survived after cryopreservation was analyzed using the chi square test. Differences were considered significant when  $P < 0.05$ .

## 3.4 Results

### 3.4.1 Epidermal growth factor and hyaluronic acid supplementation influenced embryo development, blastocyst cell number and apoptotic index

To investigate the effect of EGF and HA on embryo development, zygotes were cultured until the blastocysts stage in BM supplemented with EGF and/or HA. Supplementation of culture media with EGF, HA or EGF + HA did not affect the cleavage rate and blastocyst formation compared to the control group. Nevertheless, the day 7 blastocyst rate was significantly higher in the HA supplemented group compared to the EGF group (Table 3.3). Moreover, supplementation of HA in the culture media tended to increase the blastocyst total cell number and the ICM: TE ratio compared to

the control (Table 3.3). On the other hand, while supplementation of EGF increased, supplementation of HA during the entire preimplantation period reduced the apoptotic index in the resulting blastocysts (Figure 3.2).

Table 3.3. The development rate and blastocysts cell number of embryos cultured in presence of EGF, HA or EGF + HA throughout preimplantation period

	BM	BM + EGF	BM + HA	BM + EGF + HA
No. zygotes	425	416	466	446
Cleavage rate	81.3 ± 4.9	82.2 ± 5.9	84.4 ± 6.2	82.1 ± 3.8
Day 7 blastocyst rate	28.9 ± 7.1 <sup>ab</sup>	23.8 ± 7.08 <sup>a</sup>	32.1 ± 6.1 <sup>b</sup>	28.7 ± 5.3 <sup>ab</sup>
Day 8 blastocyst rate	42.7 ± 8.1	37 ± 8.2	42.1 ± 5.6	38.6 ± 5.6
Day 9 blastocyst rate	46.1 ± 6.4	41.5 ± 7.4	44.9 ± 5.9	39.7 ± 7.4
Blastocyst total cell number	127±7	123±13	131±4	118±6
ICM:TE ratio	1.73	1.74	1.87	1.81

Different letters (a, b) in the same row indicate significantly different ( $p < 0.05$ )

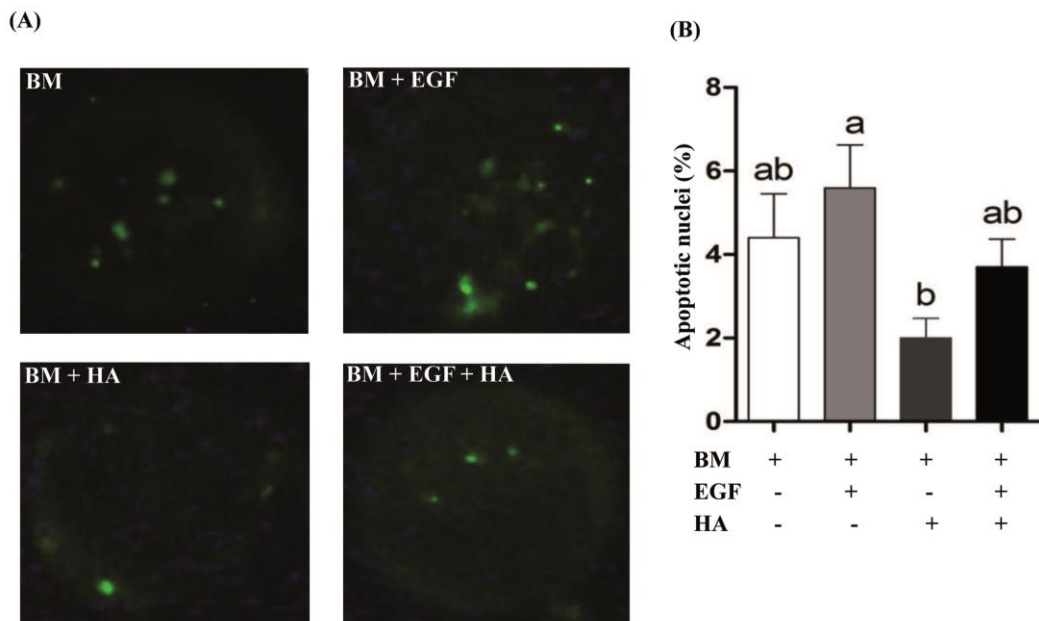


Figure 3.2. TUNEL assay in blastocysts of different blastocysts group. (A) Representative blastocysts showing TUNEL positive cells. (B) The percentage of apoptotic nuclei is presented as mean ± SEM. Bars with different letters (a, b) are statistically significant different at  $p < 0.05$

3.4.2 Epidermal growth factor and/or hyaluronic acid supplementation during in vitro embryo culture suppressed the oxidative stress and increased the cryotolerance abilities of the resulting blastocysts

In this study, the reactive oxygen species (ROS) level was significantly reduced in the blastocysts derived from zygotes cultured in culture media supplemented with EGF, HA or EGF + HA (Figure 3.3). In addition, the blastocysts derived from zygotes cultured in media supplemented with EGF or HA exhibited higher re-expansion rates at 24, 32 and 48 h after thawing compared to the control group. However, at 56 h post thawing, the re-expansion rate was significantly reduced in the HA supplemented group compared to the EGF group (Figure 3.4A). Moreover, the hatching rate was significantly higher in the treated groups at all-time points after thawing compared to the control counterparts (Figure 3.4B).

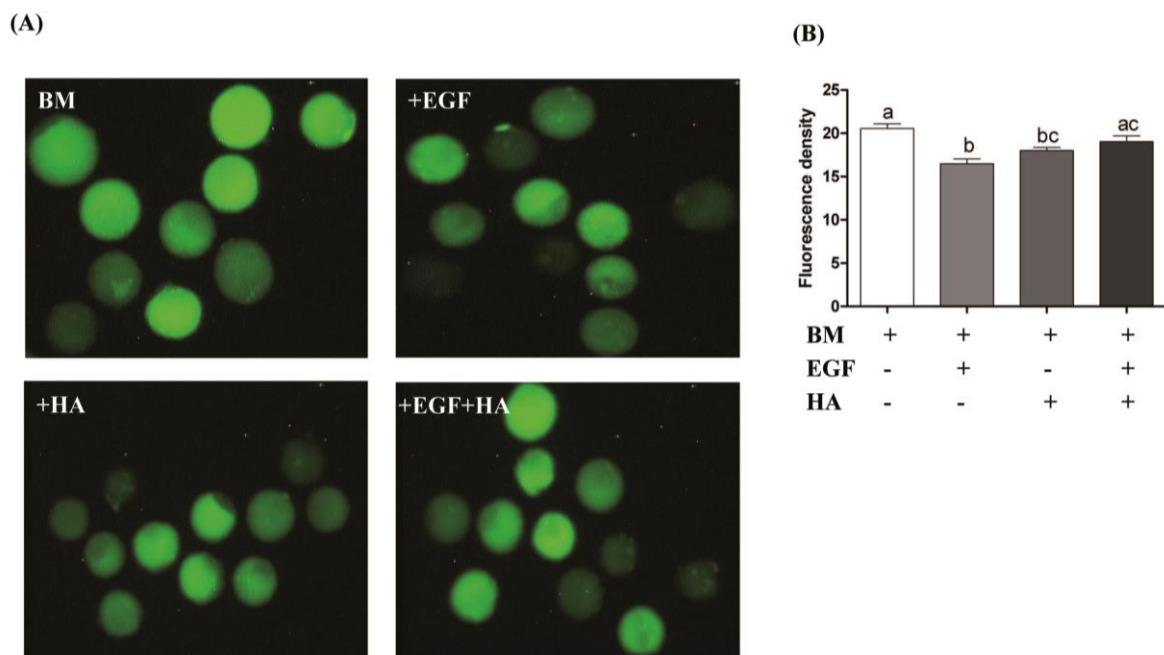


Figure 3.3. Intracellular ROS accumulation in blastocysts derived from zygotes cultured in media supplemented with EGF and/or HA. (A) Representative figures displaying the ROS accumulation levels in the blastocysts. (B) The fluorescent density (mean  $\pm$  SEM) showing the level of ROS in each blastocyst group. Bars with different letters (a, b, c) are statistically significant ( $p < 0.05$ ).

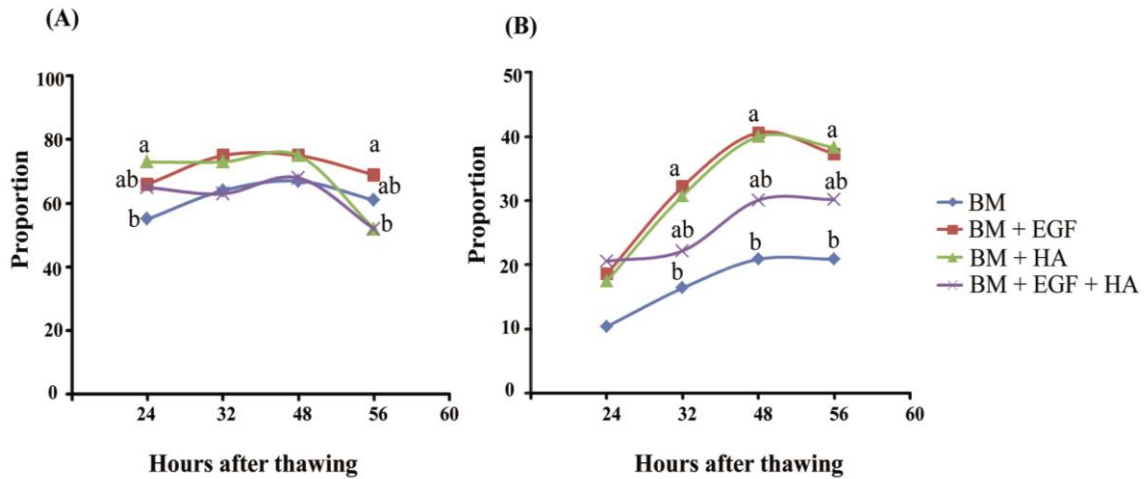


Figure 3.4. Supplementation of EGF and/or HA on the cryotolerance ability of the embryos. The expansion (A) and hatching rate (B) of blastocysts of different groups at 24, 32, 48 and 56 h after thawing. Data are presented as mean  $\pm$  SEM and different letters (a, b) on the bars indicate statistical significant differences ( $p < 0.05$ )

### 3.4.3 Epidermal growth factor and/or hyaluronic acid supplementation altered the expression of epidermal growth factor and hyaluronic acid receptor genes

In order to identify the association of EGF and/or HA supplementation and the activity of their receptors, the mRNA abundance of EGF and/or HA receptors were quantified in blastocysts derived from embryos developed in the culture media supplemented with EGF and/or HA during the entire preimplantation period. While, supplementation of HA increased the expression level of its receptor (CD44 and HMMR) genes, supplementation of EGF significantly reduced the mRNA expression level of its receptor (EGFR). However, when EGF was supplemented with HA, the expression level of EGFR was significantly increased indicating that HA could regulate the binding of EGF with its receptor (Figure 3.5).

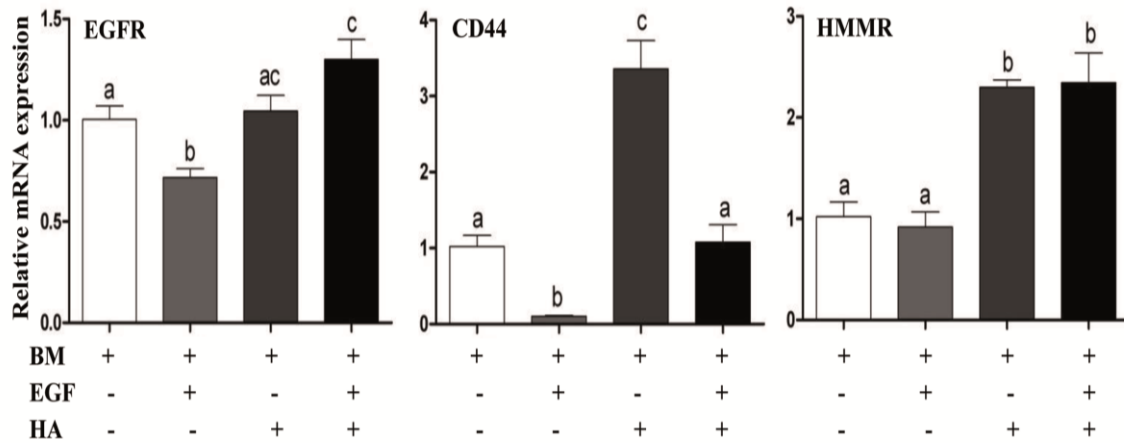


Figure 3.5. Effect of EGF and/or HA supplementation on the expression level of EGF and HA receptors. Data are presented as mean  $\pm$  SEM. Bars with different letters (a, b, c) are statistically significant ( $p < 0.05$ )

#### 3.4.4 Epidermal growth factor and hyaluronic acid supplementation induced the expression of genes involved in focal adhesion pathway

The expression level of candidate genes involved in the focal adhesion pathway was analyzed in blastocysts derived from treatment of different groups. For this, the mRNA level of the genes representing the ligands (COL1A2 and COL4A1), cytoplasmic structures (ACTG1), adaptors and focal adhesion marker (VCL), enzymatic regulation (FAK), inhibitor of focal adhesion kinase (PTEN) and actin regulators (RAC1 and PAK4) were quantified using qRT-PCR. The results revealed that except ACTG1, the expression level of the candidate genes was increased in the blastocysts derived from embryos cultured in the presence of EGF + HA during the entire preimplantation period (Figure 3.6). In addition, immunoblotting assay showed higher FAK and VCL protein expression levels in the blastocysts of EGF + HA treatment group. Furthermore, immunohistochemical analysis indicated higher VCL protein on the cell membrane in the blastocysts derived from embryos cultured in media supplemented with EGF + HA (Figure 3.7).

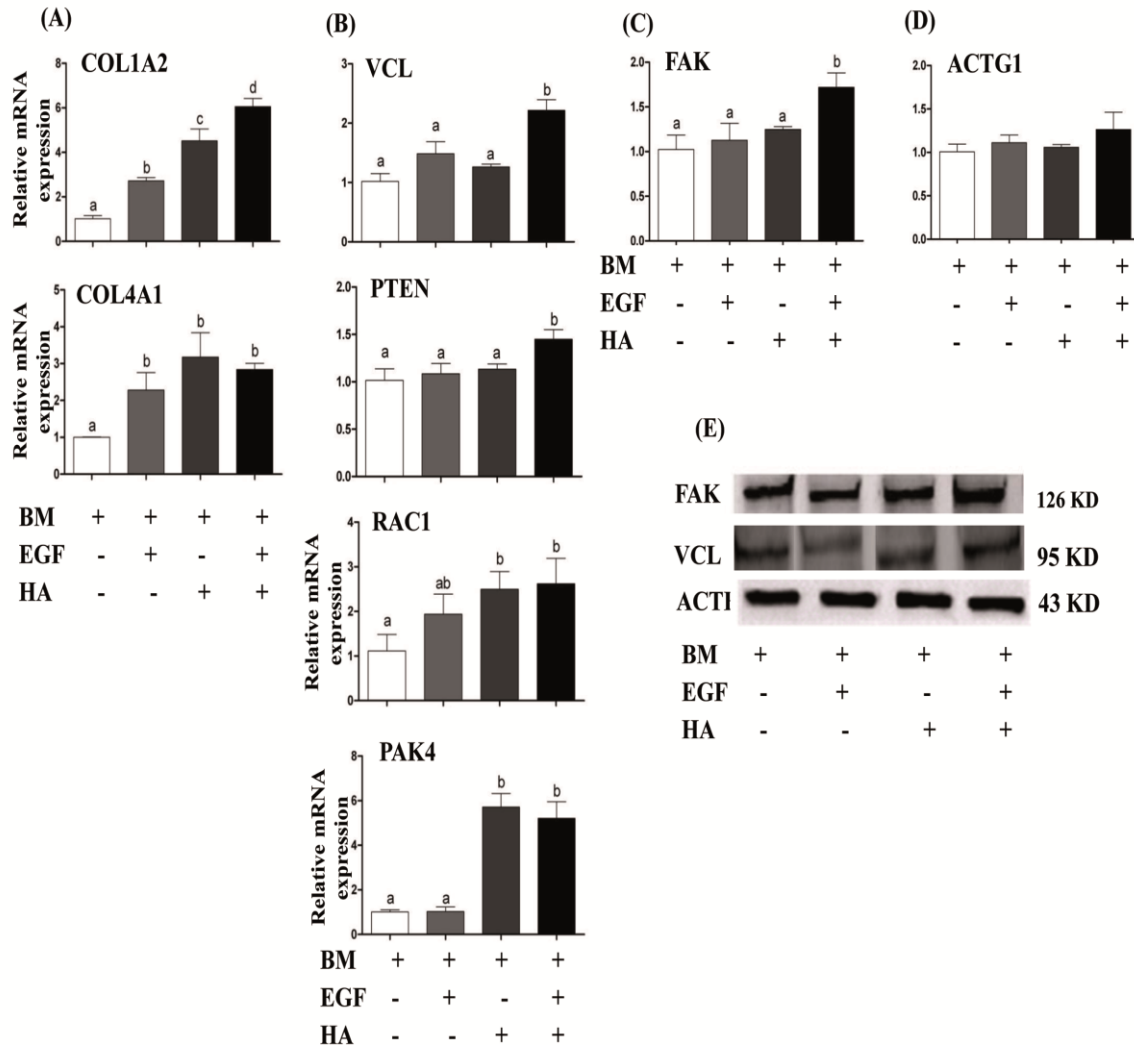


Figure 3.6. The relative mRNA expression of ligands (A), adaptor (B), regulator (C) and structure linker (D) of the focal adhesion pathway genes in blastocysts of different treatment groups. Western blot analysis of FAK and VCL genes in blastocysts derived from zygotes supplemented with or without EGF and/or HA (E). Bars represent mean  $\pm$  SEM. Different letters (a, b, c, d) on bars are statistically significant ( $p < 0.05$ )



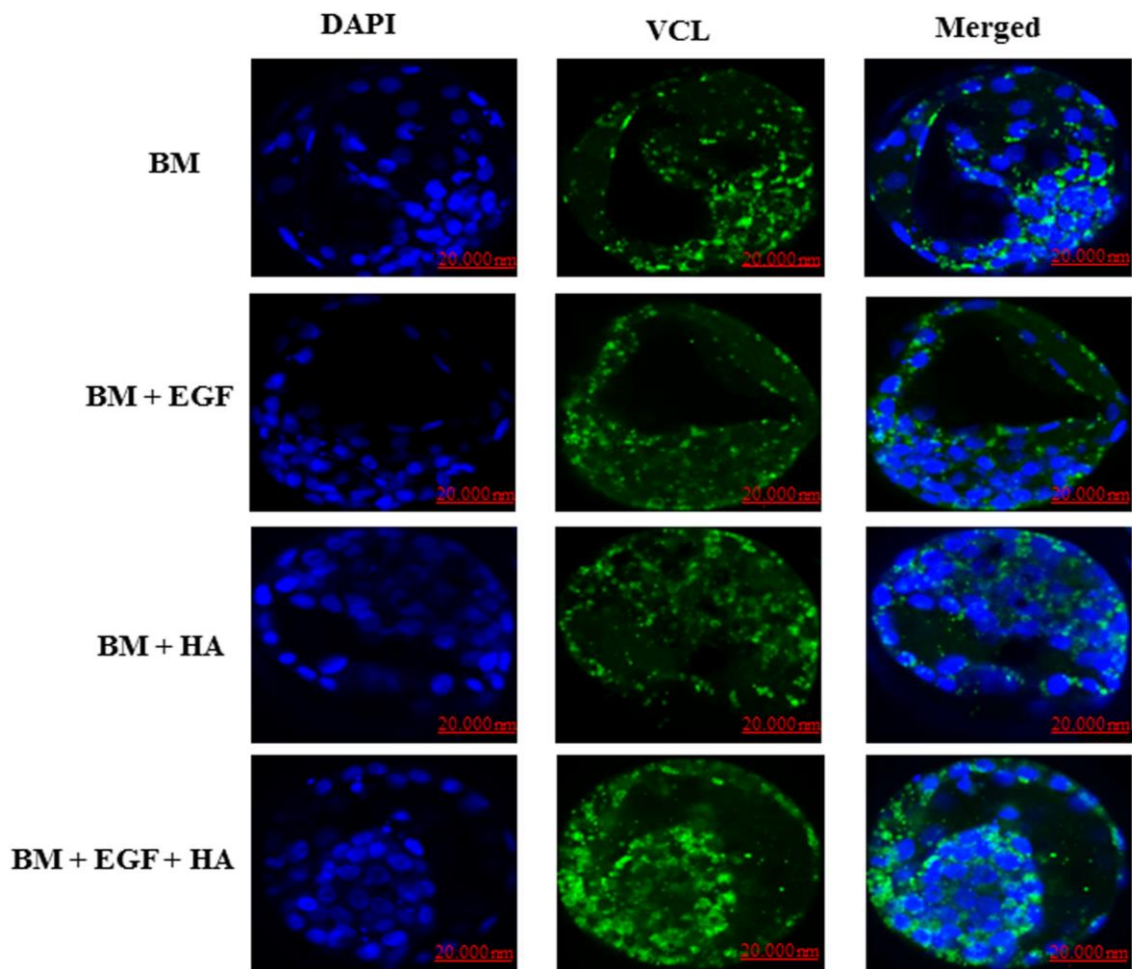


Figure 3.7. Immunohistochemical analysis of VCL, the focal adhesion protein marker gene, in blastocysts derived from zygotes cultured in different treatment groups. The green colour indicates the expression of protein, while the blue colour indicates the nuclear staining using 4',6-diamidino-2-phenylindole (DAPI)

#### 3.4.5 Combined supplementation of epidermal growth factor and hyaluronic acid altered the embryo development and expression of genes involved in focal adhesion pathway in a stage specific manner

The results from the first experiment indicated that the preimplantation embryos cultured from zygote to blastocyst stage in the presence of EGF + HA showed a significant increase in the mRNA and protein expression level of genes involved in focal adhesion pathway. However, in the first experiment, supplementation of EGF and/or HA was performed throughout the preimplantation period and it was not clear whether the effect of EGF + HA was prominent before or after the embryonic genome activation. Therefore, to address this, embryos were supplemented with EGF + HA after

4-cell (After\_4C) or after 16- cell stage (After\_16C). Other embryo groups were cultured in BM supplemented with EGF + HA until 4-cell (Until\_4C) or until 16-cell stage (Until\_16C) and then cultured only in BM until the blastocysts stage. The results revealed that the day 7 blastocysts rate were relatively higher in Until\_16C group compared to After\_16C and Until\_4C supplemented group. On the other hand, the day 8 and 9 blastocyst rates in After\_4C supplemented group tended to be higher compared to other groups (Table 3.4).

Table 3.4. The cleavage and blastocyst rate of embryos cultured in presence or absence of EGF + HA before or after embryonic genome activation

	BM	After_4C	After_16C	All_stages	Until_4C	Until_16C
Nr. zygotes	176	235	166	184	252	206
Cleavage rate	93.2±0.8	86.8±1.5	86.8±3.2	91.3±1.4	86.3±0.4	86.4±1.8
Day 7 blastocyst rate	27.8±1.5	27.6±2.5	23.3±7	28.7±1.2	23.8±7	28.6±2.6
Day 8 blastocyst rate	41.9±2.6	47.1±3.7	39.9±8.8	41.3±1.2	42.8±1.7	42.3±5
Day 9 blastocyst rate	46±1.3	55.9 ± 5	43.6±8.5	44±1.5	52.9±8.3	45.6±4.3

The gene expression analysis indicated that the expression level of the candidate genes (COL4A1, COL1A2, PAK4, PTK2 and ACTG1) tended to be higher in the blastocysts derived from After\_4C and Until\_16C supplemented group compared to After\_16C supplemented group (Figure 3.8) which was consistent with developmental data (Table 3.4). However, unlike other genes, the expression of RAC1 was increased in the blastocysts of the Until\_4C group, indicating that supplementation of EGA + HA affects the RAC1 gene expression before embryonic genome activation.

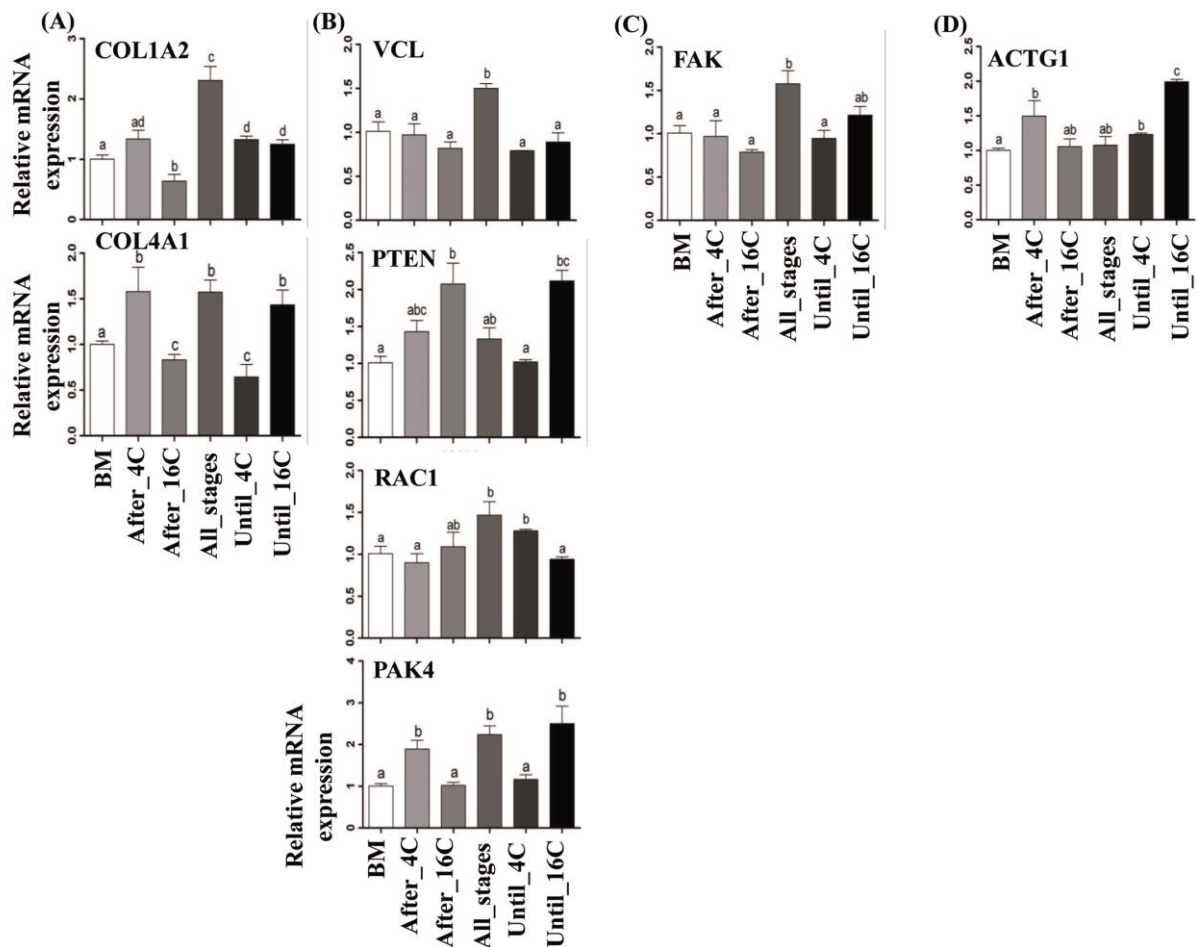


Figure 3.8. Supplementation of EGF + HA before or after embryonic genome activation on the expression pattern of ligands (A), adaptor (B), regulator (C) and structure linker (D) of focal adhesion pathway genes. Data presented as mean  $\pm$  SEM. Different letters (a, b, c, d) are statistically significant ( $p < 0.05$ )

### 3.4.6 Supplementation of epidermal growth factor and hyaluronic acid altered the DNA methylation patterns of genes involved in the focal adhesion

Since supplementation of EGF and/or HA affected both the blastocyst quality and the expression of genes involved in focal adhesion pathway, we speculated that EGF and HA may affect the embryo quality by epigenetically regulating the genes involved in the focal adhesion pathway. For this, first the expression level of genes associated with DNA methylation namely, the DNA methyltransferase genes (DNMT1, DNMT3A and DNMT3B) was investigated in the blastocysts obtained from embryos cultured in media supplemented with EGF, HA and EGF + HA from zygote to blastocyst stage or with EGF + HA pre or post embryonic genome activation. Results showed that the expression level of DNMT3A was significantly increased in the blastocysts derived from culture media supplemented with EGF and decreased in the EGF + HA group.

However, the blastocysts derived from embryos cultured in BM supplemented with EGF, HA or EGF + HA during the entire preimplantation period did not show any significant effects on the expression levels of DNMT1 and DNMT3B (Figure 3.9A). Nevertheless, the expression of DNMT1 was significantly higher in After\_4C supplemented group compared to Until\_4C supplemented ones. In addition, the expression level of DNMT3B was significantly higher in blastocyst of Until\_16C compared to After\_4C group, whereas the mRNA level of DNMT3A was significantly increased in the Until\_4C supplemented group (Figure 3.9B).

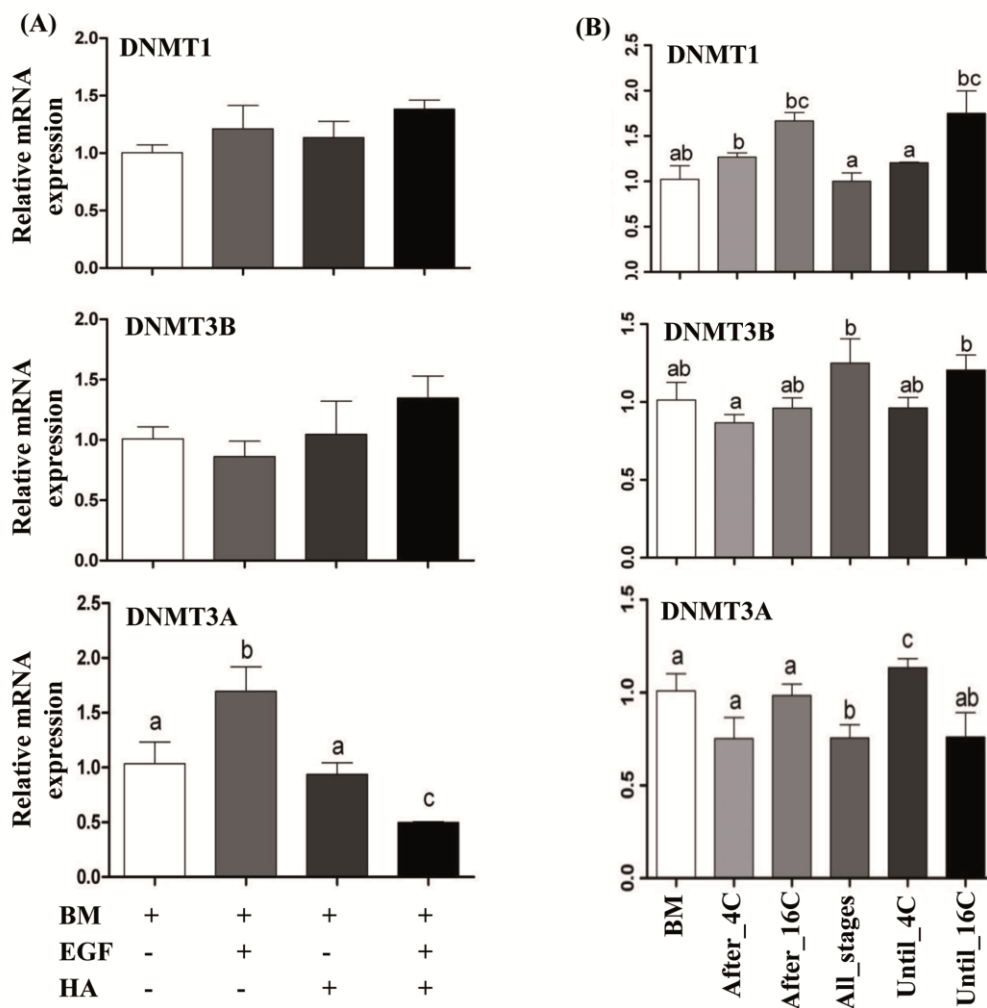


Figure 3.9. Relative mRNA expression level of DNMT1, DNMT3B and DNMT3A in blastocysts derived from zygotes cultured in media supplemented with or without EGF and/or HA until blastocyst stage (A) or blastocysts derived from embryo supplemented with or without EGF + HA before or after embryo genome activation (B). Data are presented as mean  $\pm$  SEM. Bars with different letters (a, b, c) are statistical significant ( $p < 0.05$ )

Following the expression analysis, we have performed DNA methylation analysis in three candidate genes, namely COL4A1 (with 7 CpG sites at the promoter region and 9 CpG sites at distal promoter region), COL1A2 (with 8 CpG sites at the promoter region and 22 CpG sites at the distal promoter region) and RAC1 (with 8 CpG sites at the promoter region and 14 CpG sites at the intronic region). The three genes were selected for DNA methylation analysis as their expression was affected by continued or stage specific supplementation of EGF + HA and their critical role in the focal adhesion pathway.

In vitro culture of embryos in the presence of EGF during the entire preimplantation period increased and decreased the DNA methylation level of the COL4A1 gene promoter and distal promoter, respectively, in the resulting blastocysts compared to the control group. Combined supplementation of EGF and HA increased the DNA methylation level of the COL4A1 gene both at the promoter and distal promoter regions. Apart from the entire preimplantation period, pre or post embryonic genome activation supplementation of EGF + HA indicated that the DNA methylation was highly reduced in the blastocysts derived from embryos supplemented with EGF + HA after the 16-cell stage (After\_16C) (Figure 3.10).

Similarly, the effect of EGF and/or HA on the DNA methylation pattern of COL1A2 genes at its promoter and distal promoter region was investigated using a similar methodology employed for the COL4A2 gene. Results showed that supplementation of HA alone or in combination with EGF through the preimplantation period decreased and increased the promoter and distal promoter DNA methylation level of COL4A2 gene in the resulting blastocysts. While, supplementation of EGF + HA after the 16-cell stage (After\_16C) increased the promoter methylation of COL4A2 gene in the blastocysts compared to Until\_4C or Until\_16C group but the reverse was true in the distal promoter where higher DNA methylation was detected in blastocysts derived from Until\_16C group (Figure 3.11).



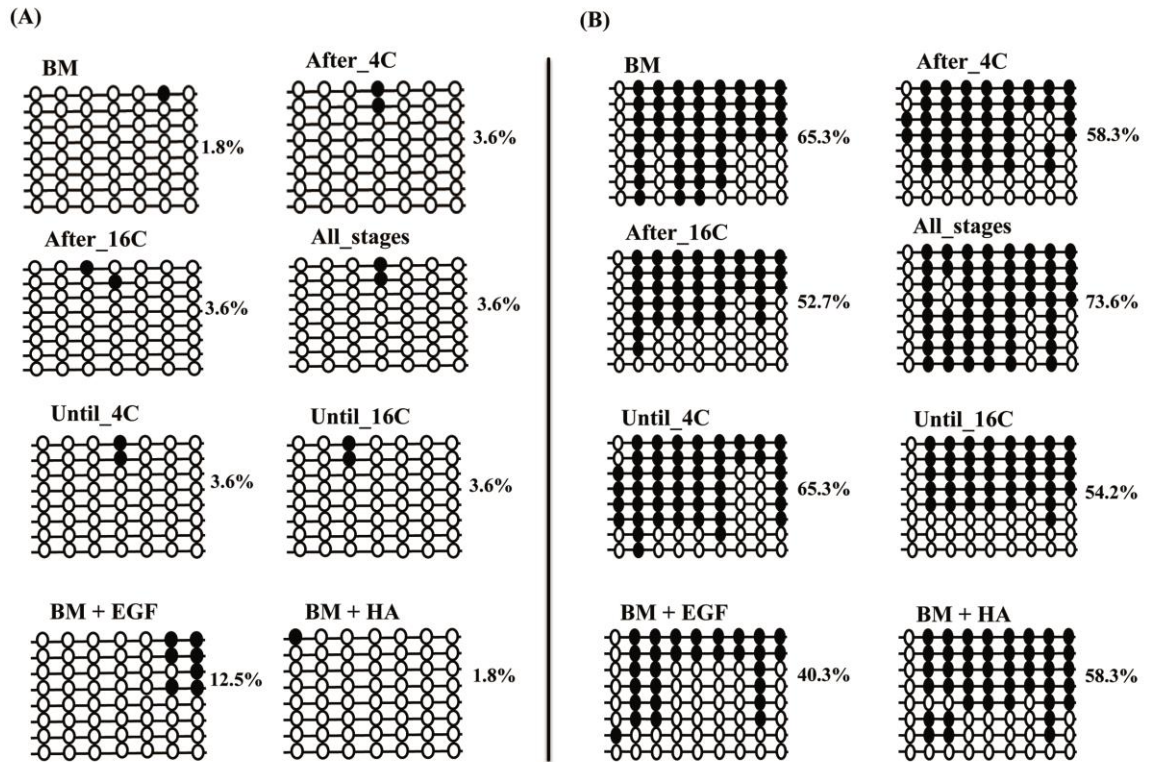


Figure 3.10. The DNA methylation level of COL4A1 at the promoter (A) or distal promoter (B) region of blastocysts of different treatment groups. The black and white circles indicate the methylated and unmethylated CpG sites, respectively

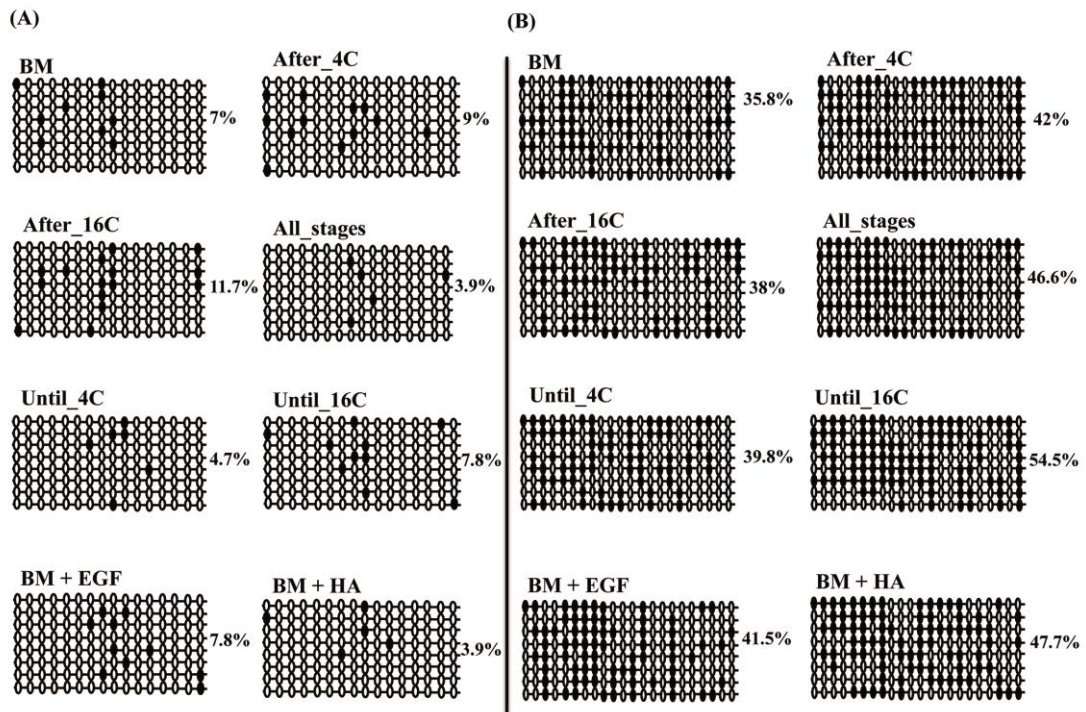
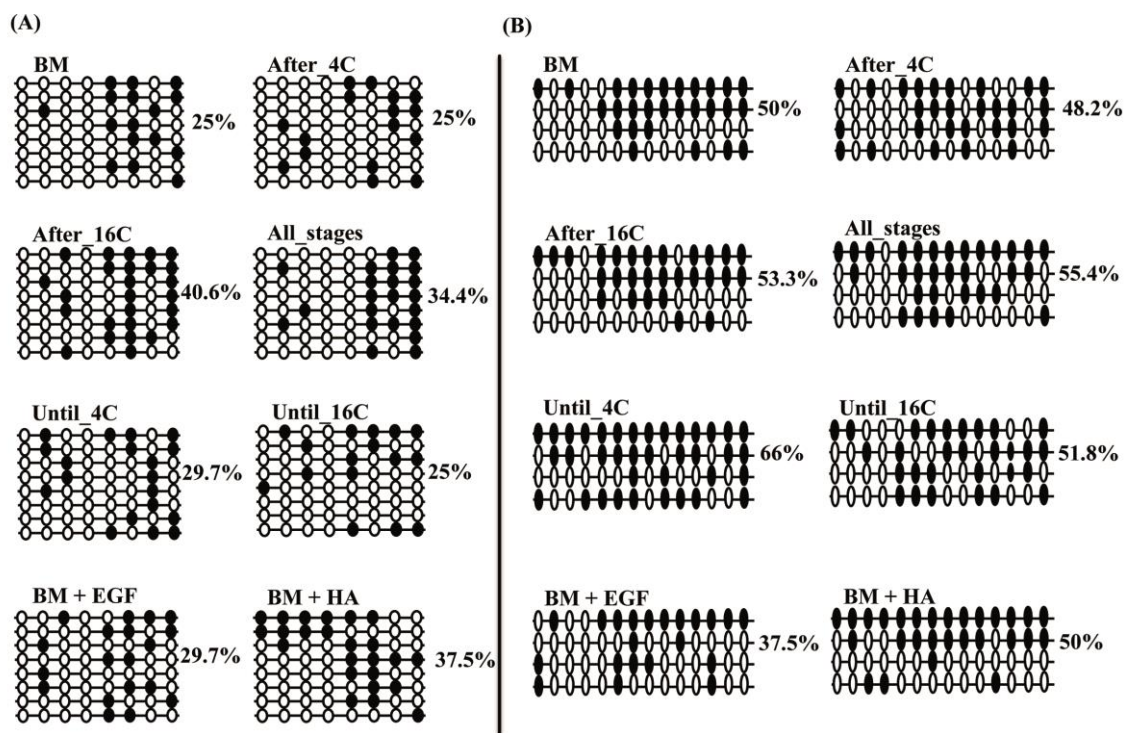


Figure 3.11. The DNA methylation level of COL1A2 at the promoter (A) or distal promoter (B) region of blastocysts of different treatment groups. The black and white circles indicate the methylated and unmethylated CpG sites, respectively

In addition, the effect of supplementation of EGF + HA on the DNA methylation pattern of the RAC1 gene was also investigated in resulting blastocysts. Accordingly, the results showed that supplementation of embryo during the entire preimplantation period with EGF alone or in combination with HA increased RAC1 gene DNA methylation at the promoter and intronic region. Moreover, the results from stage specific supplementation of EGF + HA showed that blastocysts obtained from After\_4C group were less methylated than After\_16C supplemented group (Figure 3.12). In the intronic region, the DNA methylation pattern of After\_4C and After\_16C were similar, but both were hypermethylated compared to the control and the All\_stages groups. Relatively higher DNA methylation of in the intronic region RAC1 gene was detected in the blastocysts of the Until\_4C group compared to blastocysts of all treatment groups.

Figure 3.12. The DNA methylation pattern of RAC1 gene at promoter (A) or gene body



region (B) of blastocysts of different treatment groups. The black and white circles indicate the methylated and unmethylated CpG sites, respectively

### 3.4.7 Comparative analysis of gene expression and DNA methylation

Once, we have analyzed the expression and DNA methylation patterns in blastocysts of different group, we performed comparative analysis between the expression and DNA methylation levels. For this, the DNA methylation data of COL4A1, COL1A2 and RAC1 were superimposed to the expression level. Accordingly, the results indicated the DNA methylation pattern of COL4A1 at the promoter region was negatively correlated with the gene expression patterns in blastocysts of After\_4C, After\_16C, Until\_4C and BM + HA group. However, at distal promoter of COL4A1, the DNA methylation patterns were negatively correlated with the corresponding gene expression in all treatment groups except After\_16C and All\_stages groups (Table 3.5).

Table 3.5. The correlation between the DNA methylation and expression patterns of the candidate genes in blastocysts of different groups

<b>Expression = E</b>		BM	After_4C		After_16C		All_stages		Until_4C		After_16C		BM + EGF		BM + HA		
<b>Methylation = M</b>		E	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M
COL1A2	Promoter	↓	↑	↑	↑	↓	↑	↑	↓	↑	↓	↑	↑	↑	↑	↑	↓
	Distal promoter	↓	↓	↑	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
COL4A1	Promoter	↓	↓	↑	↑	↓	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑	↓
	Distal promoter	↓	↑	↑	↓	↓	↓	↑	↑	↓	↑	↑	↓	↑	↓	↑	↓
RAC1	Promoter	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑
	Intron	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↑	↑	↓	↑	↑	↓

The symbols ↑ and ↓ indicate up and downregulation of gene expression, respectively while ↑ and ↓ indicate the hypermethylation and hypomethylation of the gene, respectively

Similarly, the DNA methylation level in the distal promoter region of COL1A2 was positively correlated with its gene expression in all blastocysts groups except in After\_4C and After\_16 groups, while the DNA methylation of COL1A2 at the promoter region was negatively correlated with the corresponding gene expression in blastocysts of After\_16C, All\_stages, Until\_4C and BM + HA groups. Moreover, at the intronic region of the RAC1, the DNA methylation pattern was positively correlated with gene expression in all blastocyst groups except After\_16C; BM + EGF and BM + HA (Table 3.5).



### 3.5 Discussion

Robust embryo-maternal communication permits normal development, high quality embryos, proper implantation and maintenance of pregnancy (Wolf et al. 2003). Thus, understanding the mechanism how the preimplantation embryos communicate with the *in vivo* or *in vitro* extracellular micro-environment is one of the most vital aspects to be considered to unravel the effects of suboptimal environmental conditions during embryogenesis. In this regard, our previous results demonstrated that blastocysts derived from *in vivo* and *in vitro* alternative culture conditions exhibited altered expression of genes and DNA methylation pattern of genes involved in focal adhesion pathway (Gad et al. 2012, Salilew-Wondim et al. 2015).

Focal adhesion is one of the essential molecular signaling pathways that regulate interaction and communication between the cell and the extracellular micro environment (Wu 2007). Therefore, focal adhesion is required for normal cell growth (Fu et al. 2004) and motility, proliferation, differentiation and migration (Wu 2007, Kaneko et al. 2012). However, little is known about the effect of suboptimal culture conditions on the expression pattern and regulation of genes involved in the focal adhesion pathway during bovine embryo development. Indeed, previous studies indicated that normal cell growth is associated with stimulation of cell adhesion in the presence of growth factors (Renshaw et al. 1999). Moreover, dysregulation of maternal or embryonic secreted-growth factors may lead to disruption in metabolism, chromosomal abnormalities, alteration of gene expression and apoptosis induction during mammalian preimplantation embryo development (Gopichandran and Leese 2006, Riedemann et al. 2007). Thus, supplementation of the culture media with growth factors such as epidermal growth factor could modulate cell growth (Goodsell 2003) and improve embryo development (Lorenzo et al. 1994, Sirisathien et al. 2003, Neira et al. 2010, Shabankareh and Zandi 2010, Prochazka et al. 2011, Song et al. 2011, Fujihara et al. 2014) by redistributing focal adhesion constituents to adhesion sites and by amplifying the levels of focal adhesion proteins members (Eberwein et al. 2015). Additionally, hyaluronic acid, an extracellular matrix component which is highly abundant in follicular, oviduct and uterine fluids in human, cattle, pig and other species (Lee and Ax 1984, Archibong et al. 1989, Suchanek et al. 1994, Ohta et al. 2001), is involved in multicellular functions including cell proliferation, differentiation, migration and apoptosis (Laurent and Fraser 1992, Kaneko et al. 2000; Toole 2001, Qhattal et al.

2014, Oommen et al. 2016, Donejko et al. 2017) by increasing the cell-to-cell communication and cell-to-extracellular matrix adhesion (Turley and Moore 1984). Supplementation of culture media with HA was found to improve bovine and porcine oocyte maturation and embryo development (Yokoo et al. 2007, Marei et al. 2012, Opiela et al. 2014). However, there is no concrete evidence regarding the effect of EGF and HA on expression and methylation pattern of focal adhesion pathway and subsequently embryonic development and quality. Therefore, in the present study, we cultured the bovine preimplantation embryos in presence or absence of EGF and/or HA to investigate whether EGF and HA could affect the bovine embryonic development and quality by affecting the expression and DNA methylation pattern of genes involved in the focal adhesion pathway. Results revealed that supplementation of culture media with 10 ng/mL EGF did not significantly affect the cleavage and the blastocyst rates. These results were consistent with previous reports (Keefer et al. 1994, Ahumada et al. 2013, Rios et al. 2015) that EGF alone or in combination with HA did not influence the bovine blastocyst rate. On the other hand, it has been reported that presence of HA during in vitro bovine embryo development resulted in an increase in blastocyst rate (Furnus et al. 2003, Marei et al. 2012, Opiela et al. 2014). In this regard, the current study indicated that addition of HA in the culture media significantly increased the day 7 blastocyst rates compared to the EGF group although the results were not significantly different when it was compared with the control group. Moreover, there was a positive effect of HA on total cell number of the blastocysts and in reducing the apoptotic cells suggesting the potential role of hyaluronic acid in regulation of cell proliferation and apoptosis (Turley and Moore 1984).

One of the critical cellular phenotypes that mark the development and quality of in vitro produced embryos is the level of intracellular reactive oxygen species (ROS) (Bedaiwy et al. 2010). In line to this, the current study revealed significant reduction of ROS level in the blastocysts obtained from embryos cultured in a media supplemented with EGF or HA or in combination of EGF and HA. Furthermore, the reduction of ROS was accompanied by reduced apoptotic index. Indeed, growth factors including EGF are believed to reduce the ROS in embryo (Kurzawa et al. 2004). Moreover, supplementation of HA could also reduce the ROS production in cells by regulating Nrf2 and Akt genes (Onodera et al. 2015). Nevertheless, the interaction between EGF and HA receptors is believed to increase the ROS level (Kim et al. 2008). Furthermore,

cryopreservability of the embryo is one of the main quality indicators of embryos developed under different culture conditions (Rizos et al. 2001, Rizos et al. 2003, Kuzmany et al. 2011). In the current study, supplementation of embryo culture media with EGF and/or HA improved the blastocyst cryotolerance. This was revealed by significantly higher expansion and hatching rate after thawing. Previous studies also showed that HA was found to improve the embryo cryotolerance and increase pregnancy rate after transfer (Gardner et al. 1999, Dattena et al. 2007, Block et al. 2009).

Following the phenotypic assessment, we have also analyzed the effect of EGF and HA supplementation on the expression patterns of genes involved in the focal adhesion pathway in blastocysts of different treatment groups. Prior to this, we have analyzed the expression level of EGF receptor (EGFR) and HA receptors (CD44 and HMMR) and a significant decrease in the expression of EGFR was detected in blastocysts derived from embryos that were cultured in media supplemented with EGF, but increased in blastocysts derived from culture with the supplementation of HA or a combination of EGF and HA. This may suggest that HA is essential for EGFR activation which could influence the binding of EGF with EGFR cell membrane receptor. Several studies demonstrated how the growth factors such as EGF interact with HA through its receptors namely, CD44 and HMMR (Jackson et al. 1991, Roy et al. 1993, Papakonstantinou et al. 1995, Tirone et al. 1997, Furnus et al. 2003). Indeed, supplementation of culture media with HA is believed to promote the rapid appearance of HMMR expression (Zhang et al. 1998). In bovine embryos, the number of HMMR receptors increased significantly at morula stage and were then reduced when embryos were cultured in serum-containing medium (Stojkovic et al. 2003). In agreement with these observations, our results showed that bovine embryos exhibited significantly high expression level of CD44 and HMMR in blastocysts derived from zygotes that were cultured in the presence of HA.

In the current study, the expression pattern of the genes involved in the focal adhesion pathway was investigated in blastocysts developed from zygotes cultured in media supplemented with EGF or /and HA. The results showed that the presence of HA or a combination of EGF and HA in the culture media increased the expression level of many of the candidate genes including the Focal adhesion kinase (FAK) gene. FAK, is a non-receptor tyrosine kinase focal adaptor protein which is localized in integrin-focal

sites (Mitra et al., 2005) to link the growth factors and integrins (Eberwein et al. 2015). The expression of FAK is induced either by direct effect of growth factors such as EGF (Eberwein et al. 2015) or by the action of extracellular signal-regulated kinase1/2 (ERK1/2) which is activated by EGF and HA stimulation (Eberwein et al. 2015, Donejko et al. 2017). When its expression is increased and actively phosphorylated, the FAK gene induces the phosphatidylinositol 3-kinase (PI3K) expression (Mitra et al. 2005), ultimately leading to the induction of the RAC1 and PAK family genes (Manser et al. 1997, Yang et al. 2011). In line with this, our results revealed a higher expression level of RAC1 and PAK4 genes in blastocysts derived from embryos cultured in the presence of HA or a combination of EGF and HA. Likewise, higher expression level of RAC1 in EGF and HA supplementation has been previously reported. Indeed, FAK and RAC1 are believed to induce focal adhesion complex proteins such as vinculin, a focal adhesion marker protein that controls focal adhesion (Memili and First 2000; Chang et al. 2007, Carisey and Ballestrem 2011). Our results also revealed higher mRNA and protein expression of vinculin in blastocysts produced under the supplementation of EGF and HA.

Once we have realized the effect of supplementation of EGF and/or HA throughout the preimplantation period, we opted to determine whether these effects were pronounced before or after embryo genome activation. By considering the fact that major bovine EGA occurs around the 8- to 16-cell stages (Memili and First 2000, Graf et al. 2014) and the minor genome around the 4-cell embryonic stage of development, we have generated blastocysts from zygotes cultured in the presence or absence of EGF + HA until 4 or 16-cell stages or presence or absence of EGF + HA after 4- or 16-cell stages. Accordingly, the day 7 blastocyst rate was relatively lower in embryos supplemented with EGF + HA after 16- cell stage or supplemented only until-4 cell stage compared to those supplemented throughout the preimplantation period or supplemented until 16-cell stage suggesting EGF + HA may be beneficial during the time of embryonic genomic activation. Similarly, the gene expression analysis also indicated an increase in the expression level of the focal adhesion pathway genes in blastocyst groups derived from embryos supplemented with EGF + HA from 4-cell stage until blastocyst stage or from zygote stage until 16-cell stage indicating the importance of EGF + HA during the time of embryonic genome activation. We have also analyzed the DNA methyltransferase genes to get insight about whether the genes responsible for DNA methylation activity

could be altered by supplementation of EGF and/or HA during embryo development. The expression level of DNMT3A, which is one of the key genes which involved in de novo DNA methylation, was significantly affected either by continuous supplementation throughout the preimplantation development or pre and post embryonic genomic activation supplementation. Similarly, previous data also indicated higher expression of DNMT3A in bovine embryos cultured under different in vitro culture conditions (Sagirkaya et al. 2006). Therefore, alteration of DNMT3A in the current study may indicate the possible DNA methylation changes induced by these culture conditions. According to that, in the current study, the DNA methylation pattern of candidate genes was analyzed in blastocysts developed under continued supplementation of EGF + HA until the blastocyst stage or supplemented only before after embryonic genome activation. Bisulfite sequencing data revealed that the DNA methylation pattern of candidate genes (COL1A2, COL4A1 and RAC1) was changed by continuous or pre- and post EGA supplementation of EGF and/or HA on the promoter, distal promoter or gene body region and the results are in agreement with our previous findings reported in in vitro- in vivo alternative embryo culture conditions (Salilew-Wondim et al. 2015). Moreover, the DNA methylation analysis in blastocysts of different groups demonstrated that, the DNA methylation at the promoter regions of COL4A1, COL1A2 and RAC1 genes was induced in the EGF group compared to the HA ones, but the gene expression tended to be lower in the EGF group compared to the HA group. Moreover, significantly higher day 7 blastocyst rate and lower apoptotic index was observed in HA group compared to the EGF ones suggesting that the DNA methylation and gene expression at the promoter regions of COL4A1, COL1A2 and RAC1 could be associated with bovine embryo development and blastocyst quality. When we compare the EGF + HA supplemented with control group, the hatching rate of the blastocysts after thawing was improved in the supplemented group where the gene expression was also increased. Furthermore, the DNA methylation pattern of the candidate genes was inversely or positively correlated to the gene expression in the promoter, distal promoter or in the gene body regions. For instance, the DNA methylation at the promoter region of COL4A2 and RAC1 was negatively correlated with the gene expression in blastocysts developed under HA, EGF or EGF + HA supplemented media from zygote to the blastocyst stage. Moreover, including the, the distal promoter methylation patterns of the COL4A1 gene were negatively correlated

with the gene expression. However, the DNA methylation pattern in the gene body region of the RAC1 gene was positively correlated with gene expression in all blastocyst groups except After\_16C, BM + EGF, BM + HA.

In conclusion, this was the first study that demonstrated the effect of culture media supplemented with EGF and/or HA throughout preimplantation or pre and post embryonic genome activation on development and quality of bovine preimplantation embryos, expression and DNA methylation pattern of the focal adhesion pathway. Supplementation of HA resulted in a better blastocysts rate than EGF. Nevertheless, ROS accumulation of the blastocyst was significantly reduced and cryotolerance was increased by supplementation of EGF and/or HA. This was accompanied by an increase in the expression of genes involved in the focal adhesion pathway and changes in the DNA methylation pattern. Moreover, the results revealed the importance of supplementation of growth factors such as epidermal growth factor and extracellular components such as hyaluronic acid during the time of bovine embryonic genome activation.

### 3.6 Declarations

#### Ethics approval and consent to participate

The study was conducted on bovine in vitro produced blastocysts using oocytes collected from slaughterhouse ovaries and thus special approval of this experiment was not required.

#### Consent for publication

Not applicable.

#### Availability of data and material

All data used and/or analyzed during the present study are available from the corresponding author if requested.

#### Competing interests

The authors declare that they have no competing interests.

#### Funding

Not applicable.

#### Authors' contributions

MSZ, DT, MH, DSW and KS designed the experiments; KS and DT supervised the experiments; MSZ, MH, EV and FR performed in vitro embryo production; MSZ performed lab experiments involving mRNA, protein, DNA methylation analysis; MSZ, DSW and ET analyzed the data; MSZ, and DSW interpreted the data; MSZ drafted the manuscript; DSW critically reviewed and edited the manuscript; KS, DT, MH, YMS and CN reviewed the manuscript. All authors approved the final version of the paper.

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Chapter 4: General discussion

#### 4.1 Discussion

The prominent role of mammalian ovary is to produce competent oocytes through repeated folliculogenesis. The follicular development is a coordinated and periodic process, which initiates with activation of resting follicles leading to growth and selection of dominant follicle from small follicles accompanied with sequential and profound differentiation of oocyte and the surrounding somatic cells, especially granulosa cells (Zhang et al. 2017). Granulosa cells are playing a vital role throughout the folliculogenesis including oocyte growth and development followed by ovulation and fertilization. For instance, the complicated transition process from primordial to matured follicles is controlled by granulosa cells. Hence, the proliferation, apoptosis, cell-cell communication and remarkable functional differentiation of granulosa cells are considerable events and required for follicle maturation (Tajima et al. 2006). Therefore, it is indispensable to understand the molecular mechanism and pathways involved in the complex orchestrated process of forming the competent oocyte and embryo. Meanwhile, the quality of oocyte is crucial factor of successful pregnancy in farm animals. Sartori et al. (2010) reported that around one-third of embryonic death during the first week after fertilization is associated with poor oocyte quality in dairy cows. The importance of oocyte quality is due to maternal factors, that are stored and accumulated in the oocyte during the course of folliculogenesis and oogenesis and they are essential for maternal-to-embryonic transition (Zhang and Smith 2015). The maternal-to-embryonic transition consists of critical developmental processes including maternal RNA depletion, epigenetic reprogramming/chromatin remodeling and embryonic genome activation (Bettegowda et al. 2008). Furthermore, embryogenesis in the period from fertilization to implantation involves various morphological, cellular, and biochemical changes related to genomic activity (maternal and embryo genome) (Stanton et al. 2003). The time of embryonic genome activation is species specific which occurs at the two-cell stage in mouse (Schultz 1993), at the four-cell stage in human (Braude et al. 1988), and at the late eight-cell stage in bovine embryos (Memili and First 2000, Graf et al. 2014). Therefore, environmental effects on the genome and epigenome must be investigated in order to improve animal production.

Communication of oocyte as well as the preimplantation embryo either with the maternal or in vitro surrounding environment is essential for normal growth and subsequently high pregnancy rate. Cell communication with extra cellular environment

via mechanisms including cell-extracellular matrix adhesion including focal adhesion and extracellular vesicles like exosomes is among of the fundamental communication mechanisms that alter intra and extracellular responses signals. However, clear evidence is still elusive regarding to the effect of suboptimal conditions including oxidative stress on exosomal content of mRNAs cargo related to oxidative stress and the potential role of exosomes in horizontal genetic transfer of antioxidant molecules that influence the oxidative stress tolerance in bovine granulosa cells and subsequently oocyte improvement. Additionally, a little is known regarding to epigenetic regulatory mechanisms including DNA methylation in the regulation of focal adhesion pathway and subsequently development and quality of preimplantation embryos cultured under different culture media. Therefore, this study was built up on two major experiments to roll out the potential role of exosomes in oxidative stress defense mechanism in bovine follicular granulosa cells and the role of DNA methylation in the focal adhesion pathway regulation in bovine preimplantation embryos.

Here, we exposed in vitro cultured bovine granulosa cells to oxidative stress conditions induced by moderate level of  $H_2O_2$  (Chapter 3) and thereafter, exosomes were isolated from culture media of treated and untreated groups 24 hr post-treatment. Hydrogen peroxide ( $H_2O_2$ ) is one of reactive oxygen species (ROS) that is continuously generated in live cells during cellular metabolism under aerobic conditions, it is known that the addition of  $H_2O_2$  can induce oxidative stress and cell death since it effectively pervades through the cell membrane (Chernyak et al. 2006). Several studies have demonstrated the regulation of oxidative stress response at the transcriptional level (Kops et al. 2002, Omata et al. 2008, Szybowska et al. 2011) that can be mediated by microvesicles including peroxisomes and exosomes, where exosomal shuttle of RNA can mediate a signal during oxidative stress response (Valadi et al. 2007, Eldh et al. 2010). Results from this experiment revealed that oxidative stress induced intracellular ROS accumulation was accompanied with low mitochondrial activity and cell proliferation rate and this was ensured by shifting in cell cycle profile of stressed granulosa cells compared with non-treated cells (Chapter 2, Figures 2.11, 2.2 and 2.3). ROS including superoxide anion ( $O^{2-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $\cdot OH$ ) have a critical physiological role in mammalian folliculogenesis and ovulation process; however, excess ROS level leads to oocyte and granulosa cells dysfunction (Tamura et al. 2008). Mammalian follicular degeneration is determined by the regulation of



granulosa cells survival and apoptosis (Shiota et al. 2003). Differentiation of granulosa cells into luteal cells occurs by a surge of ovulatory luteinizing hormone (LH), which stimulates the expression of StAR protein, a rate-limiting step for progesterone synthesis (Hickey et al. 1988, Ronen-Fuhrmann et al. 1998, Lee et al. 2013). On the other hand, during ovulation and after the LH surge, ROS are locally produced within the follicle by endothelial cells, neutrophils and macrophages (Brannstrom and Norman 1993), suggesting that granulosa cells are exposed to elevated levels of ROS that can lead to granulosa cells apoptosis (Tanabe et al. 2015). Redundant granulosa cells apoptosis is responsible for follicular atresia (Devine et al. 2012), and subsequently ovarian dysfunction (Qu et al. 2012).

Excessive level of ROS can damage cellular components including nuclei, mitochondria and plasma membranes, resulting in malfunctioning of DNA, loss of membrane integrity and mitochondrial dysfunction; the latter in particular is often related to apoptosis (Halliwell 2001, Nagy 2001, Mishra and Dhali 2007, Wu et al. 2011). Moreover, H<sub>2</sub>O<sub>2</sub> resulted in induction of pro-apoptotic genes and reduction of anti-apoptotic related genes (Chapter 2, Figure 2.6), which was also showed by other studies conducted on human granulosa cells (Yang et al. 2017) and resulted in reduction of cell proliferation (Chapter 2, Figure 2.3) as previously reported (Eldh et al. 2010). Cell response to oxidative stress via several mechanisms among them is Nrf2-mediated oxidative stress response, which is the most important defense mechanism (Akino et al. 2017). Nrf2 is a transcriptional factor that partly control in cytosol by Keap1; however, under stress conditions Nrf2 is translocated to nucleus and activate antioxidant enzymes to protect against and eliminate the effects of stress (Nguyen et al. 2009). Our results showed higher cellular mRNA and protein levels of Nrf2 and its downstream antioxidants (PRDX1, HMOX1, CAT, SOD1, TXN1 and NQO1) in bovine granulosa cells challenged with H<sub>2</sub>O<sub>2</sub> compared to control group (Chapter 2, Figures 2.4 and 2.5). Similar results have been recently revealed the similar mRNA alteration on Nrf2 and downstream antioxidant as a response to H<sub>2</sub>O<sub>2</sub> treatment in human granulosa cells (Akino et al. 2017).

Exosomes are well known as nano-sized (30-150 nm) extracellular vesicles (El Andaloussi et al. 2013) and were discovered in the early 1980s (Pan et al. 1985). Exosomes were found to be released from all cell and fluid types that have been studied so far (Théry et al. 2002) and contain a cargo of mRNAs, small and long non coding

RNAs, proteins, lipids and DNA that can be horizontally transferred to extracellular space as well as near or long distance neighboring cells (Valadi et al. 2007, Muralidharan-Chari et al. 2010). They have fundamental role in cell-to-cell communication and involve in several biological processes (Théry et al. 2002). However, environmental factors influence exosomes properties and their content. For that, exosomes are sensitive to any in vivo or in vitro changes or stresses (Théry et al. 2006, Beninson and Fleshner 2014). Depending on how and where exosomes were released, they can have a plenty of effects (Eldh et al. 2010). Our results of nanoparticle tracking and electron microscope indicated that oxidative stress conditions resulted in high concentration and large size of exosomes (StressExo) compared with control one (NormalExo) (Chapter 2, Figure 2.7), it was reported that there is diversity in exosomes quality, quantity and function under different pathological conditions, and different culture conditions resulting in different exosomal mRNA content (Eldh et al. 2010, Yuan et al. 2016). Exosomal total RNA of StressExo was enriched with Nrf2 and its downstream CAT and TXN1, along with a lower mRNA level of PRDX1 and HMOX1 genes as compared with NormalExo (Chapter 2, Figure 2.8).

Horizontal transfer of genetic information via exosomes has been reported by several studies (Lee et al. 2012), they contain different functional factors, which decide their properties, either harmful or beneficial to recipient cells (Navakanitworakul et al. 2016). Our results showed that StressExo were higher in their exosomal mRNA level of Nrf2, CAT and TXN1 genes. Under normal or stress conditions the co-culture of granulosa cells with NormalExo or StressExo showed higher mRNA and protein levels of Nrf2 and its downstream antioxidants compared to those of cultured under normal conditions. However, under stress conditions the co-culture of granulosa cells with StressExo showed significantly higher cellular mRNA and protein levels of Nrf2 and its downstream antioxidants compared with other groups (Chapter 2, Figures 2.13 and 2.14), which in turn led to reduction in intracellular ROS level and subsequently increased mitochondrial activity under stress conditions (Chapter 2, Figures 2.10 and 2.11). It is well known that Nrf2 and its downstream antioxidants control the intracellular ROS level (Kovac et al. 2015). Accordingly, these findings revealed the horizontal transfer of oxidative stress response elements via exosomes under stress conditions and their roles in inducing cellular oxidative tolerance. Co-incubation with StressExo induced granulosa cell proliferation through influencing the cell cycle profile

(Chapter 2, Figure 2.12). Several studies have revealed the beneficial effects of exosomes uptake (Braccioli et al. 2014) and the protective effects of exosomal mRNA shuttle against oxidative stress (Eldh et al. 2010). Moreover, co-incubation of bovine embryos with exosomes enhanced embryonic development (Qu et al. 2017).

In the second experiment, we focused on another signaling mechanism (focal adhesion pathway) involved in cell communication to extracellular matrix (ECM) under suboptimal culture conditions. In this scenario, we aimed to investigate the effect of different culture media (Chapter 3, Figure 3.1) supplemented with EGF and/or HA on expression and regulation of focal adhesion pathway through DNA methylation pattern and subsequently development and quality of bovine preimplantation embryos. Adhesion of cells to ECM components and the presence of growth factors are essential for normal cell growth and functions (Renshaw et al. 1999, Lovicu et al. 2011). Focal adhesion is known as integrin-mediated mechanical link between intracellular actin cytoskeleton and ECM (Wu 2007). Focal adhesion involves in several biological functions such as cell survival, migration, proliferation, motility and differentiation (Romer et al. 2006, Ivaska and Heino 2010) as well as during oocyte and embryo development (Wu 2007, Ivaska and Heino 2010, Wolfenson et al. 2013) and subsequently implantation and pregnancy (Bloor et al. 2002). Stable and normal focal adhesion is required for normal cell growth (Fu et al. 2004), however, there are several factors alter focal adhesion signaling. It has been reported in several cell types that the FAK is cleaved during cells exposed to stress (Crouch et al. 1996, Levkau et al. 1998). Furthermore, induction of ROS accumulation leads to cleavage of FAK which in turn results in acutely and chronically apoptosis induction in HeLa S cells (Mian et al. 2008). In human fibroblast, oxidative stress induced by ethanol has a negative effect on FAK in a dose-dependent manner. This effect could be reversible by applying hyaluronic acid via its positive role on the expression and activation of FAK (Donejko et al. 2017). On the other hand, focal adhesion signaling pathway is one of the most dominant downregulated genes due to exposing of bovine mammary epithelial cells to heat stress (Li et al. 2015). It was reported that integrin signaling is one of the dominant pathways in bovine embryos exposed to in vivo and in vitro alternative culture conditions at 4 and 16-cell stages (Gad et al. 2012). However, the effect of environmental stresses on the focal adhesion in bovine reproduction and their relationship with infertility is still unclear and need more investigations in order to provide concrete view for

manipulation. The current study was the headmost investigation to provide a new window to highlight the on the effect of sub-optimal culture media supplemented with EGF and HA on the mRNA expression and DNA methylation pattern of genes involved in focal adhesion pathway of bovine preimplantation embryos.

Here, we designed our experiment in such a way to culture the preimplantation embryos in four culture groups of media supplemented with EGF and/or HA. In addition, these factors have their specific cell membrane receptors independent of integrins. EGF has specific membrane receptor (EGFR) and is one of the main growth factors for cell proliferation in many cell types (Wong and Guillaud 2004). EGF was found to improve the development rate during early stages of mouse (Adamson 1993), porcine (Wei et al. 2001), bovine (Cebrian-Serrano et al. 2014) embryos. The second factor is HA as main component of ECM has its specific receptors (HMMR and CD44), it has reported that supplementation with HA improved blastocyst rate in bovine in vitro produce embryos (Block et al. 2009). For that, we cultured bovine zygotes until blastocyst stage in SOF media (BM) supplemented with EGF 10 ng/ml and/or HA 1 mg/ml. The results showed higher mRNA expression level of genes involved in focal adhesion pathway of blastocysts derived from zygotes supplemented with EGF and/or HA compared to control group. Moreover, HA alone or combined with EGF had significantly higher mRNA expression level of EGF and HA receptor genes as well as genes involved in focal adhesion pathway compared to control and EGF groups (Chapter 3, Figures 3.5 and 3.6). The protein analysis results of FAK and VCL proteins as focal adhesion markers (Chang et al. 2007, Carisey and Ballestrem 2011) showed the same mRNA pattern in each blastocyst group, moreover immunohistochemistry assay showed high cell membrane localization of VCL protein in HA and EGF+HA groups (Chapter 3, Figures 3.6 and 3.7). EGF has been reported to stimulate cell proliferation and motility through activation of MAP-kinase/ERK2 (Hauck et al. 2000). Additionally, another evidence suggested that EGF response underlies an EGFR integrin cross-talk with the induction of FAK and Src, as well as MAP kinase (Eberwein et al. 2015) and the inhibition of EGFR and FAK resulted in cell apoptosis (Golubovskaya et al. 2002). On the other hand, HA influences focal adhesion through FAK protein activity (Hall et al. 1994, Donejko et al. 2017).

The induction of focal adhesion pathway as a result of EGF and/or HA supplementation was in correspondence with embryo development and quality, which was confirmed by

improvement of day 7 blastocyst rate accompanied with higher cell number and lower apoptotic cells in HA group (Chapter 3, Table 3.3 and Figure 3.2). Furthermore, EGF and HA groups showed reduction of intracellular ROS level with high expansion and hatching rates after embryo cryopreservation (Chapter 3, Figures 3.3 and 3.4). A considerable number of evidences revealed the positive effect of EGF on oocyte and embryo competence and development (Lonergan et al. 1996, Sirisathien et al. 2003, Ahumada et al. 2013). In contrast, our development data did not show any positive effect of EGF on blastocyst rates, which could be a result of poly unsaturated fatty acids absence in culture media used in our study (Sylvester et al. 1994, Cowing and Saker 2001). However, HA alone (Stojkovic et al. 2002, Palasz et al. 2006, Opiela et al. 2014) or a combination of EGF and HA (Rios et al. 2015) was found to improve oocyte maturation and embryo development. Furthermore, EGF and/or HA enhanced embryo cryopreservation and induced hatching rate after freeze-thawing (Lane et al. 2003, Block et al. 2009), which could be a result of EGF and HA protective effect against stress (Donejko et al. 2017), the regulation of Nrf2 and activation of Akt (Kurzawa et al. 2004, Onodera et al. 2015).

Additionally, in order to determine the effective time-point of EGF and HA on focal adhesion pathway, preimplantation embryos were cultured at different developmental stages (4-cell, 16-cell and all-stages) with or without a combination of EGF and HA. Gene expression analysis showed higher mRNA expression level of genes involved in focal adhesion pathway in blastocyst derived from zygotes culture in media supplemented with a combination of EGF and HA after 4-cell or until 16-cell stages or throughout all stages (Chapter 3, Figure 3.8). These results revealed that the presence of EGF and HA during embryonic genome activation period (8 to 16-cell stage in bovine) was significantly effective on the expression level of focal adhesion genes. Our previous results showed a high number of differentially expressed genes occurred in blastocysts flushed from in vivo to in vitro culture conditions before embryonic genome activation, while a lower number of differentially expressed genes was noticed in blastocysts transferred from in vitro to in vivo conditions before embryonic genome activation (Gad et al. 2012). According to the aforementioned results, we hypothesized that, the focal adhesion pathway alters as a result of different culture media (BM, +EGF, +HA and +EGF+HA) through epigenetic regulatory mechanisms including DNA methylation.

DNA methylation is the most common epigenetic mechanism refers to addition of methyl group (CH<sub>3</sub>) to the cytosine nucleotide in the position of cytosine-guanine via the activity of methyltransferase enzymes (DNMT1, DNMT3A and DNMT3B) (Bird 1986, Jin et al. 2011). DNA methylation is known to be associated with the gene repression. Our previous study revealed that alternative culture conditions resulted in global DNA methylation changes in bovine preimplantation embryos that showed higher hyper and hypo methylated probes in in vitro produced embryos compared with in vivo counterparts (Salilew-Wondim et al. 2015). In parallel to mRNA expression analysis of genes related to focal adhesion pathway, we also quantified mRNA expression level of methyltransferases genes (Chapter 3, Figure 3.9) including DNMT1, which responsible for maintaining DNA methylation, and DNMT3A and 3B, which are responsible for de-novo methylation (Jin et al. 2011). Results demonstrated that neither EGF nor HA affected mRNA expression level of DNMT1 and DNMT3B. However, a significant higher mRNA level of DNMT3A was observed in blastocysts under EGF treatment, while a significant low mRNA level was in HA and EGF+HA groups compared to control one. Previous results in rabbit showed no differences at mRNA level of DNA methyltransferases genes under different culture conditions at different development stages (Salvaing et al. 2016). While, DNMT3A showed higher mRNA expression in bovine embryos cultured under different in vitro culture conditions (Sagirkaya et al. 2006). On the other hand, supplementation of EGF+HA until 4-cell stage showed an increase in DNMT3A level while, embryos that were supplemented with EGF+HA until 16-cell stage showed an increase in DNMT3B level. Changes in DNA methyltransferases regarding to change culture conditions may clarify the changes occurred in gene expression under the same time point.

Depending on gene expression analysis data from both experiments under study, COL1A2, COL4A1 and Rac1 genes were selected for DNA methylation analysis. Bisulfite sequencing data revealed that, DNA methylation pattern of these genes was differed under different culture conditions, which was in agreement with our previous results towards alternative culture conditions (Salilew-Wondim et al. 2015). Groups with high percentage of DNA methylation of Rac1 was associated with decreased mRNA level in contrast to COL1A2 and COL4A1, which showed hypo-methylation pattern and high mRNA expression level. These findings were agreement with previous studies that reported negative correlation between DNA hyper-methylation in promoter

site and mRNA expression of the gene (Huang et al. 2014). However, hyper-methylation occurred in distal promoter of COL1A2 and COL4A1 was accompanied with increased expression level (Chapter 3, Figures 3.10, 3.11 and 3.12). Interestingly, it is believed that hyper and hypo-methylation in distal promoter regulate the gene expression (Thomas 1993). Furthermore, alternative culture conditions before and after EGA led to changing of DNA methylation pattern compared to in vivo counterparts (Salilew-Wondim et al. 2015). In this line, our results showed that the supplementation of EGF and HA during EGA was associated with DNA methylation pattern that accompanied with high mRNA expression.

#### 4.2 Conclusion and future prospective

In conclusion, our study revealed the potential roles of extracellular vesicles including exosomes and focal adhesion pathway in cell-cell communication through horizontal genetic transfer and via epigenetic regulation of cell adhesion signaling pathway. Taken together, our investigations opened up new insights to further investigations in order to understand genetic and epigenetic mechanisms during oocyte and embryo development. Therefore, future experiments could be directed towards investigating:

- Molecular mechanisms involved in exosome selectivity to carry a specific molecule under different suboptimal conditions
- Correlation between focal adhesion pathway and exosome secretion in bovine follicular cells and embryos under different culture conditions
- Cross-talk between DNA methylation profile and exosomes secretion
- Intra tissue and intra species horizontal transfer of genetic information via exosomes and its implication on folliculogenesis and embryo development

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## 6. Research publications:

Peer reviewed published articles

1. **Saeed-Zidane M**, M.S.Abdel-Fattah, H.S.Khamis, A.M.El-Sherbiny and E.E.Tharwat (2009). Effect of Fed Crushed Date Palm on Thyroid Activity and Metabolic Changes of Weaned Barki Lambs in Siwa Oasis". Research Bulletin, Ain Shams Univ.1-16.
2. M.S. Abdel-Fattah, Afaf A. Abdel-Hamid, Ashgan M. Ellamie, M.M. El-Sherief and **M.S. Zidane (2012)**. Growth Rate, Some Plasma Biochemical and Amino Acid Concentrations of Barki Lambs Fed Ground Date Palm at Siwa Oasis, Egypt. American-Eurasian J. Agric. & Environ. Sci., 12, 166-1175.
3. 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 M-A, 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.
4. E.Held-Hoelker, S.L.Klein, F.Rings, D.Salilew-Wondim, **M.Saeed-Zidane**, C.Neuhoff, D.Tesfaye, K.Schellander, M.Hoelker (2017). Cryosurvival of in vitro produced bovine embryos supplemented with l-Carnitine and concurrent reduction of fatty acids. Theriogenology, 96: 145-152.
5. 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, 11: e0187569.

Peer reviewed articles under process

1. Hyaluronic acid and epidermal growth factor improved the bovine embryo quality by regulating the DNA methylation and expression patterns of the focal adhesion pathway. **Mohammed Saeed-Zidane**, Dawit Tesfaye, Yousri Mohammed Shaker, Ernst Tholen, Christiane Neuhoff<sup>1</sup>, Franca Rings<sup>2</sup>, Eva Held<sup>1,2</sup>, Michael Hoelker, Karl Schellander, Dessie Salilew-Wondim (Submitted to BMC Genomics).
2. Suboptimal culture condition before minor and major embryonic genome activation strongly affects the DNA methylation landscape of resulting bovine blastocysts. Dessie Salilew-Wondim, **Mohammed Saeed-Zidane**, Michael Hoelker,

Samuel Gebremedhn, Michael, M. Poirie, Harri Om Pandya, Ernst Tholen, Christiane Neuhoff, Urban Besenfelder, Vita Havlicek, Franca Rings, Eric Fournier, Dominic Gagné, Marc-André Sirard, Claude Robert, Karl Schellander, Dawit Tesfaye (Submitted to BMC Genomics).

3. Intrafollicular transfer of abattoir-derived in vitro-matured or immature oocytes results in blastocysts' gene expression profiles comparable to fully in vivo-produced blastocysts. Mohammad Bozlur Rahman, Md Aminul Islam, **Mohammed Saeed-Zidane**, Franca Rings, Dessie Salilew-Wondim, Dawit Tesfaye, Karl Schellander, Michael Hölker (Under submission).

#### Conference abstracts

1. **Saeed-Zidane M**, Salilew-Wondim D, Rings F, Hoelker M, Neuhoff C, Tholen E, Looft C, Schellander K, Tesfaye D (2015): Regulation of genes related to focal adhesion pathway during bovine preimplantation embryo development. Vortragstagung der DGfZ und GfT, 16./17.9.2015, Berlin, Germany, Tagungsband: D18 (Abstr, Oral presentation)

2. **Saeed-Zidane M**, Salilew-Wondim D, Linden L, Held E, Neuhoff C, Hoelker M, Schellander K, Tesfaye D (2016): Exosome mediated oxidative stress response in bovine granulosa cells. Vortragstagung der DGfZ und GfT, 20./21.9.2016, Hannover, Germany, (Abstr, oral presentation).

3. **Saeed-Zidane, M.**, Salilew-Wondim, D., Neuhoff, C., Hölker, M., Schellander, K. & D. Tesfaye (2016): Regulation of focal adhesion pathway genes in bovine preimplantation embryos, 27.-29-10.2016, ESDAR Conference 2016, Lisbon, Portugal (Abstr, Oral presentation).

4. **Saeed-Zidane M**, Salilew-Wondim D, Linden L, Held E, Neuhoff C, Hoelker M, Schellander K, Tesfaye D Exosome-mediated oxidative stress response in bovine granulosa cells (2017), 16.01.2017, IETS conference, Texas, USA (Abstr, Poster).

5. **Saeed-Zidane M**, Salilew-Wondim D, Held E, Rings F, Neuhoff C, Tholen E, Hoelker M, Schellander K, Tesfaye D. DNA methylation mediated regulation of focal adhesion pathway in bovine preimplantation embryos Vortragstagung der DGfZ und GfT, 20/21.9.2017, Stuttgart, Germany, (Abstr, oral presentation).

6. M. O. Taqi, **M. Saeed-Zidane**, D. Salilew-Wondim, F. Rings, M. Hölker, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye. 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).

7. D. Salilew-Wondim, D. Tesfaye, M. Hoelker, S. Gebremedhn, E. Held, **M. Saeed-Zidane**, M. Poirier, E. Fournier, E. Tholen, C. Neuhoff, U. Besenfelder, V. Havlicek, F. Rings, D. Gagné, M. A. Sirard, C. Robert, K. Schellander. The DNA methylation changes induced in bovine blastocyst developed in vivo followed by in vitro culture condition. Vortragstagung der DGfZ und GfT, 20/21.9.2017, Stuttgart, Germany, (Abstr).

8. M. O. Taqi, S. Gebremedhn, **M. Saeed-Zidane**, D. Salilew-Wondim, F. Rings, M. Hölker, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye 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).

#### Awards

Egyptian higher education scholar grant for doctoral students (2012)

#### Skills

1. Wet lab skills: Expert with animal blood samples collection and blood parameter (hematology and hormone) analysis techniques; cell, oocyte and preimplantation embryo culture; molecular genetics, epigenetics (DNA methylation and microRNA) and protein (immunoblotting, immunocytochemistry and immunohistochemistry) analysis techniques; exosomes isolation and analysis techniques and microarray technique.
2. Dry lab skills: statistical analysis tools (SAS and GraphPad Prism); mRNA and Protein analysis software; DNA methylation analysis software and Microarray data analysis using R program.