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Modulation of Nrf2-mediated oxidative stress response in bovine granulosa cells and preimplantation embryos

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Omar Zainelabdeen Shehata Khadrawy

aus

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Dedicated to my beloved parents, my lovely brother, sister, my darling wife and my children Abdallah & Noureen

Modulation of Nrf2-mediated oxidative stress response in bovine granulosa cells and preimplantation embryos

Nrf2 is a redox sensitive transcription factor involved in regulation of antioxidants defense mechanism against various stressors. Maintaining reactive oxygen species (ROS) within cellular homeostatic levels is crucial for the female reproductive performance and ovarian ability to develop a competent oocyte capable to be fertilized and developed to competent embryo. However, Nrf2 is found to be regulated at transcriptional and posttranscriptional levels and various epigenetic mechanisms including DNA methylation, histone modifications and miRNAs. Therefore, this dissertation aims to assess the potential role of noncoding miRNAs as endogenous and quercetin as exogenous regulators of Nrf2 pathway in bovine granulosa cells and preimplantation embryos. For this, two main experiments were conducted; where bovine granulosa cells were used for modulation of miRNAs (miR-28, 153 and miR-708) targeting the bovine Nrf2 and supplementation of quercentin to investigate the regulatory mechanisms of the Nrf2 antioxidant system. In addition, cultured cells exposed to oxidative stress conditions induced by hydrogen peroxide (H_2O_2) in those cells. Cells of all experimental groups were subjected to ROS level, mitochondrial activity and cell proliferation assays as well as mRNA and protein expression analyses. In the second experiment, bovine embryos were cultured in media supplemented with quercetin under high oxygen tension (20 %). Blastocysts were subjected to ROS level, mitochondrial activity, mRNA expression and protein analysis. Overexpression of miR-153, miR-28 and miR-708 reduced the expression pattern of Nrf2 at transcriptional and translational levels under physiological and oxidative stress conditions. Moreover, overexpression of miR-153 and miR-28\708 showed higher ROS accumulation, lower mitochondrial activity and cellular proliferation. Furthermore, quercetin supplementation in culture media showed a protective role on bovine granulosa cells and preimplantation embryos against oxidative stress induced by H_2O_2 and high oxygen level, respectively through activation of Nrf2 machinery leading to reduce ROS accumulation, increased mitochondrial activity and cellular proliferation and embryonic total cell number. These findings highlighted the regulatory mechanisms of Nrf2 mediated oxidative stress response pathway in bovine granulosa cells, preimplantation embryos and indicate the potential application of those regulatory mechanisms in future fertility treatment strategies to enhance ovarian functionality and embryo quality under suboptimal conditions.

Beeinflussung der Nrf2-vermittelten oxidativen Stressreaktion in bovinen Granulosazellen und Präimplantationsembryonen

Nrf2 ist ein redoxsensitiver Transkriptionsfaktor, der an der Regulierung des antioxidativen Abwehrmechanismus gegen verschiedene Stressoren beteiligt ist. Die Regulation der reaktiven Sauerstoffspezies (ROS) innerhalb der zellulären Homöostase ist entscheidend für die weibliche Reproduktionsleistung und die Fähigkeit der Eierstöcke kompetente Eizellen zu entwickeln, die befruchtungsfähig sind und sich zu kompetenten Embryonen entwickeln. Allerdings wurde festgestellt, dass Nrf2 auf transkriptioneller und posttranskriptioneller Ebene sowie durch verschiedene epigenetische Mechanismen - DNA-Methylierung, Histonmodifikationen und miRNAs - reguliert wird. Daher ist das Ziel der Dissertation die potenzielle Rolle von nicht-kodierenden miRNAs als endogene und die von Quercetin als exogene Regulatoren des Nrf2-Signalwegs in bovinen Granulosazellen und Präimplantationsembryonen zu bewerten. Dazu wurden zwei Hauptexperimente durchgeführt in denen bovine Granulosazellen eingesetzt wurden, um zum einen die Beeinflussung von Nrf2 Ziel-miRNAs (miR-28, miR-153 und miR-708) und zum anderen die regulierende Wirkung von Quercetin auf das Nrf2 antioxidative System zu untersuchen. Darüber hinaus wurden die kultivierten Zellen durch Wasserstoffperoxid (H_2O_2) oxidativen Stressbedingungen ausgesetzt. Mit den Zellen aller experimentellen Gruppen wurden ROS-, mitochondriale Aktivitäts- und Zellproliferationsassays sowie mRNA- und Proteinexpressionsanalysen durchgeführt. Im zweiten Experiment wurden Rinderembryonen mit Quercetin versetztem Medium und in einer hohen Sauerstoffumgebung (20 %) kultiviert. Mit den Blastozysten wurden ebenfalls ROS-, mitochondriale Aktivitäts-, mRNA- und Proteinexpressionsanalysen durchgeführt. Die Überexpression von miR-153, miR-28 und miR-708 reduzierte die Nrf2 Expression auf transkriptioneller und translationaler Ebene unter physiologischen und oxidativen Stressbedingungen. Darüber hinaus zeigte die Überexpression von miR-153, miR-28 und miR-708 eine höhere ROS-Akumulation, eine geringere mitochondriale Aktivität sowie eine geringere zelluläre Proliferationsrate. Des Weiteren konnte gezeigt werden, dass Quercetin im Kulturmedium eine schützende Wirkung auf bovine Granulosazellen und Präimplantationsembryonen vor oxidativem Stress hat, auch bei einer H_2O_2 Belastung und einem hohen Sauerstoffgehalt. Dies erfolgte jeweils durch die Aktivierung des Nrf2-Mechanismus sowie der daraus resultierenden Reduktion der ROS-Akkumulation und der

Erhöhung der mitochondrialen Aktivität sowie der zellulären Proliferation und der embryonalen Gesamtzellzahl. Diese Ergebnisse heben zum einen die Regulationsmechanismen des Nrf2-vermittelten oxidativen Stresssignalwegs in bovinen Granulosazellen und Präimplantationsembryonen hervor und zeigen zum anderen die mögliche Anwendbarkeit dieser Mechanismen in zukünftigen Fertilitätsbehandlungsstrategien, um die Funktionalität der Eierstöcke und die embryonale Qualität unter suboptimalen Bedingungen zu verbessern.

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ACTB	β-actin
BSA	Bovine serum albumin
CAT	Catalase
CCK-8	Cell-counting kit-8
cDNA	Complementray DNA
CO_2	Carbondioxide
COCs	Cumulus oocyte complexes
Ct	Threshold cycle
DAPI	4',6-Diamidin-2'-phenylindoldihydrochlorid
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
D-PBS	Dulbecco's phosphate-buffered saline
E. coli	Escherichia coli
EGF	Epidermal growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
IVF	In vitro fertilization
IVP	In vitro production
Keap1	Kelch like ECH associated protein 1
MAPK	Mitogen-activated protein kinases
miRNA	Micro RNAs
mRNA	Messenger RNA
ncRNA	Noncoding RNA
NQO1	NAD(P)H quinone dehydrogenase 1
Nrf2	Nuclear factor, erythroid 2 like 2
OD	Optical density
PBS	Phosphate-buffered saline
PBS-CMF	Ca^{2+}/Mg^{2+} free 1x phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium iodide

PRDX1	Peroxiredoxin 1
qRT-PCR	Quantitative real-time polymerase chain reaction
Que	Quercetin
ROS	Reactive oxygen species
rpm	Revoulution per minute
SEM	Standard error
siRNA	Small interfering RNA
SOD1	Superoxide dismutase 1
SOF	Synthetic oviductal fluid
TCM	Tissue culture media
UTR	Untranslated region
V/V	Volume per volume

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Chapter 2

differences (p<0.05).

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Chapter 1
General overview

1.1 Introduction

In vitro embryo production (IVP) is one of assisted reproductive technologies (ART), which provides a wide knowledge of molecular and cellular response controlling early embryonic development and quality (Nandi et al. 2006). Many essential developmental processes such as the cleavage, embryonic genome activation, morula compaction and blastocyst formation occur during the course of development post-fertilization. The in vitro culture systems have great impact on preimplantation embryo metabolism (Absalón-Medina et al. 2014), embryonic genome activation (Gad et al. 2012) and change embryonic transcription pattern (Rizos et al. 2002; Gad et al. 2012; Leite et al. 2017). Recently, it was demonstrated that Nrf2-mediated oxidative-stress-response pathway is one of the dominant responsive cascades in embryos produced under alternative culture conditions in vivo or in vitro (Gad et al. 2012). The central gene within this pathway is the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2), which is considered to be a key transcription factor that regulates a battery of 250 genes involved in a variety of cellular functions mainly cytoprotective genes against endogenous and environmental stressors (Chanas et al. 2002; Kwak et al. 2003; McMahon et al. 2001; Hayes and Dinkova-Kostova 2014). Moreover, the quality of the preimplantation embryos produced under high oxygen level was associated with the activity of Nrf2 signaling pathway as a response to oxidative stress conditions (Amin et al. 2014). Nrf2 was found to be epigenetically regulated by various mechanisms including DNA methylation, histone modifications and interactions of microRNAs (miRNAs) (Guo et al. 2015). Thus, it seems that in certain circumstances miRNA (miR)-based mechanisms are playing a role in prolonged, non-acute, changes in Nrf2 activity.

MicroRNAs are class of endogenous short non-coding RNAs (ncRNA) (18-24 nucleotides in length) that involved in the posttranscriptional regulation of genes through mRNA degradation and\or inhibition of target gene translation (Bartel 2004; Bartel 2009). Reports indicated that miRNAs are involved in the regulation of various reproductive functions including folleculogenesis (Hossain et al. 2012; Lei et al. 2010; Bahrami et al. 2017), oocyte maturation (Gilchrist et al. 2016; Sinha et al. 2017), and early embryonic development (García-López and del Mazo 2012; Yuan et al. 2016; Yang et al. 2016). Moreover, supplementation of mammalian cells and embryo culture media with antioxidant components exert a protective effect against subsequent oxidative stress

conditions throughout activating antioxidant machinary (Hosseini et al. 2009; Poljšak and Fink 2014; Kurutas 2016).

Quercetin is a flavonoid component that exerts an antioxidant effect (Naderi et al. 2003; Yang et al. 2014; Kim et al. 2006; Bao et al. 2017), accompanied with the activation of Nrf2 signaling pathway (Murakami et al. 2008; Ji et al. 2015; Ghanim et al. 2011). Thus, here we studied the involvement of miRNAs as regulatory network of Nrf2 in bovine granulosa cells and in vitro cultured preimplantation embryos under oxidative stress conditions or co-incubated with quercetinAn overview regarding to effect of oxidative stress on mammalian reproduction, cellular response, Nrf2 regulation and quercetin as potential antioxidant and Nrf2 activator are highlighted in the following sections.

1.2 Oxidative stress and mammalian reproduction

Stress is an environmental or management changes that alter internal homeostasis (Badinga et al. 1985), and inability of animals to cope these changes reflected as a failure to achieve genetic potential (Dobson and Smith 2000), which resulted in hazardous effect on animal production and reproduction (Khansari et al. 1990). In animals, stress is a reflex reaction developed when the animals are exposed to unfavorable environmental conditions resulting in undesirable effects ranging from discomfort to death as a result of oxidative stress damage (Etim et al. 2013). Oxidative stress occurs due to the lack of balance between the reactive oxygen species (ROS) and the cellular ability to counteract their effect by cellular antioxidants defense mechanisms (Persson et al. 2014). The most important biological ROS molecules can be divided to radical species such as hydroxyl radical (OH^-), superoxide anion radical (O_2^-), peroxyl (ROO^-), alkoxyl (RO^-) andhydroperoxyl (HO_2^-) and non-radical species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1O_2$) (Halliwell 1987; Rizzo et al. 2012).

Mammalian female reproduction depends on adequate development and maturation of female gamete passing through a complex of essential events such as fertilization, embryo development and implantation, which are connected to numerous signaling pathways to ensure the highest quality offspring (Ye et al. 2005; Chen et al. 2011). The reproductive process is characterized by alterations in metabolism and energy consumption accompanied by excessive production of highly active free radicals during physiological process of oxygen consumption (Fujii et al. 2005). Oxidative stress has a double sword

edge in the regulation of female reproductive system, either by controlling of different physiological functions or involved in of female infertility (Agarwal et al. 2005). Several studies have been demonstrated the physiological importance of ROS in mammalian reproduction, which is involved in folliculogenesis, oocyte maturation, corpus luteum and uterine function (Agarwal et al. 2008). Similarly, ROS has crucial role in male reproductive processes such as sperm capacitation (Chen et al. 2013), hyperactivation (Suarez 2008), acrosomal reaction (Lamirande and O'Flaherty 2008), normal sperm-oocyte interaction, embryogenesis, embryonic implantation and fetoplacental development (Lamirande et al. 1997; Agarwal et al. 2008). Excessive ROS production is associated with different cellular damage such as oxidative damage to cell membranes, DNA, mitochondria and ultimately accelerates cell death either by apoptosis or necrosis (Alvarez 2003), which may affect oocyte competence and embryo development resulted in embryo retardation (Figure 1.1; Agarwal et al. 2003). Likewise, high ROS level resulted in oxidative damage in the male germ cells lead to impaired fertilization, embryo development accompanied with pregnancy loss (Baker and Aitken 2005).
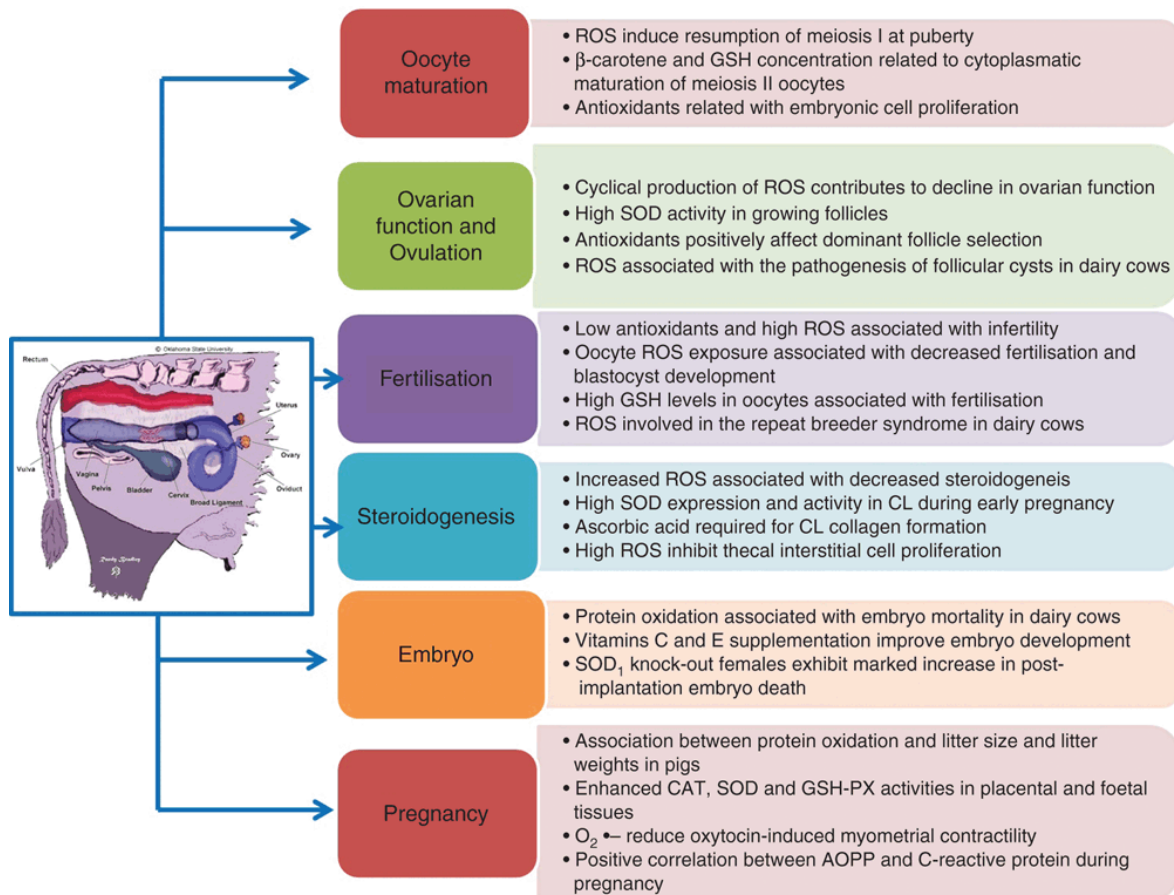
Figure 1.1. Impact of oxidative stress in female reproduction and fertility. Adapted from Talukder et al. (2017).

1.2.1 Oxidative stress in folliculogenesis, oocyte maturation and ovulation

During folliculogenesis, estrogen production rises under influence of follicle stimulating hormone (FSH), which triggers the generation of catalase (CAT) in dominant follicle to avoid apoptotic effect of ROS (Behrman et al. 2001). Moreover, granulosa cells exhibit increased metabolic activity (Sugino 2005) and steroid production due to increased activity of P450 enzymes accompanied by ROS production (Sugino 2005; Ruder et al. 2009; Rizzo et al. 2012). The dominant follicle released a mature ovum as a result of LH surge, which was found to be related to induction of inflammatory related genes (Richards et al. 2002; Gérard et al. 2004; Liu et al. 2009). These inflammatory genes found to be associated with ROS generation (Shkolnik et al. 2011). Depletion of these inflammatory genes affects ovulation process (Shkolnik et al. 2011). The corpus luteum, which developed after ovulation, generates ROS as a result of macrophage activity (Sugino et al. 1996) and luteal

cell activity (Kato et al. 1997), which affect the progesterone production (Sawada and Carlson 1996). Antioxidant deficiency mainly SOD1 was associated with reduced fertility as a result of early embryonic loss (Ho et al. 1998). Moreover, SOD1 deficiency resulted in increasing intracellular ovarian ROS accompanied with increasing apoptosis in corpus luteum and impaired progesterone production (Noda et al. 2012). SOD immunostaining showed a higher concentration in cumulus cells (Tamate et al. 1992), suggesting the protective role of cumulus cells in oocyte protection against oxidative stress through activation of antioxidants (Tatemoto et al. 2000). Furthermore, both enzymatic and non-enzymatic antioxidants are produced by granulosa cells, growing and ovulatory follicles to protect themselves against oxidative damage of ROS (Rizzo et al. 2012). Increasing intracellular ROS accumulation resulted in inhibition of oocyte maturation due to meiotic arrest (Tatemoto et al. 2000). On the contrary, another study reported that antioxidants inhibit oocyte maturation (Takami et al. 1999). Thus, it can be implied that maintaining equilibrium between ROS production and antioxidant system is critical for proper maturation of oocyte.

1.2.2 Oxidative stress during IVP of mammalian embryos

1.2.2.1 Endogenous source of ROS

ROS originates endogenously as natural by-products through multiple mechanisms depending on the cell and tissue types. The primary sites of intracellular ROS include the endoplasmic reticulum (ER), xanthine oxidase, mitochondrial electron transport chain (ETC), NADPH oxidase complexes (NOXs) and peroxisomes (Figure 1.2; Cantu-Medellin and Kelley 2013; Brand 2016). Mitochondrial membrane potentials and enhanced proton-motive forces increase ROS formation (Murphy 2009; Holmstrom and Finkel 2014). Almost 1–2 % of the electrons flow through the (ETC) in mitochondria within complexes I and III (Harris 1968), which is a primary site of superoxide production (Brand 2010). ROS is produced in complex I during reverse electron transport, where electrons enter complex I through coenzyme Q binding (Murphy 2009). Mitochondrial complex III catalyzes the electron transfer from ubiquinol to cytochrome c, which is coupled to proton translocation for ATP synthesis (Chen and Zweier 2014). Thus, increasing metabolic rate is associated with elevating the ROS production (Finkel and Holbrook 2000). The endoplasmic reticulum is a well-orchestrated protein-folding machine and sensors for the detection of

presence of misfolded or unfolded proteins. Protein folding and refolding are ATP dependent processes and ATP depletion induced ER stress can stimulate mitochondrial oxidative phosphorylation to increase the generation of ATP, ultimately further forming ROS (Malhotra and Kaufman 2007) and also ER stress can lead to the unfolded protein response (UPR) leading to calcium leakage from the ER into cytosol, then triggering ROS production in mitochondria (Csordas and Hajnoczky 2009). In peroxisomes, H_2O_2 was formed as a result of electron transfer from different metabolites to the oxygen within the respiratory pathway (Duve and Baudhuin 1966). Moreover, other free radicals were produced in peroxisomes through different peroxisomal enzymatic reactions (Schrader and Fahimi 2006).

During embryonic development, embryo needs high energy, which is met by generating ATP through aerobic metabolism including oxidative phosphorylation and glycolysis (Thompson et al. 2000). There are several enzymatic systems involved in the process of generation of ATP and subsequently raised ROS levels including oxidative phosphorylation, NADPH oxidase and xanthine oxidase systems (Guerin et al. 2001). The embryos can generate multiple forms of free radicals such O_2^-, H_2O_2, and OH^- (Manes and Lai 1995). Different stages of embryo development have varied levels of ROS generation. In mouse embryos, ROS is generated twice, at the time of fertilization and the G_2/M phase of the second cell cycle (Nasr-Esfahani and Johnson 1991). It has also been reported that the generation of ROS is not always dependent on the mitochondria, in which inhibition of mitochondrial respiration does not reduce the generation of ROS in rabbit blastocysts (Manes and Lai 1995), which indicates that there are other sources responsible for ROS generation during oxygen metabolism. NADPH oxidase was another oxidizing system produce free radicals, which is reported in the rabbit preimplantation embryos (Manes and Lai 1995). Inhibition of NADPH oxidase system can prevent generation of H_2O_2 in 2-cell mouse embryos (Nasr-Esfahani and Johnson 1991). Purine metabolism can also be responsible for the production of ROS and is associated with the developmental arrest in vitro mouse embryo (Nureddin et al. 1990).
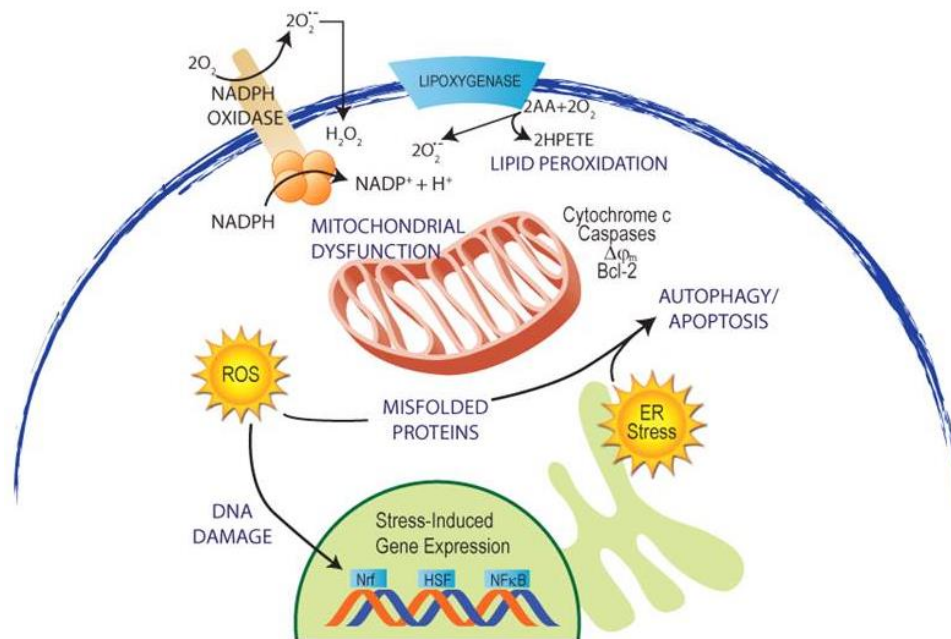
Figure 1.2. Endogenous sources of ROS in mammalian cells adapter from https://http://www.enzolifesciences.com

1.2.2.2 Exogenous source of ROS

On the other hand, the external sources of ROS are plenty and the techniques of IVP itself also contribute to the generation of ROS. Among the external factors the oxygen concentration, visible light, amine oxidase, media and additives, spermatozoa, excess glucose, metallic ions, freeze-thaw process, and many pollutants are reported to cause excessive ROS production (Figure 1.3). The ROS levels have been reported to be raised in vitro culture as compared to in vivo counterparts mouse embryo (Goto et al. 1993). It is well known that the in vitro system has more oxygen concentration than the in vivo situation. Several studies have clarified the effect of oxygen level in the culture system during the IVP in different species. For instance, the embryo developmental competence, in terms of blastocyst formation, was significantly higher in the embryos cultured under low oxygen concentration (5% O_2) compared to embryos generated under higher oxygen concentration (20% O_2) in mouse (Orsi and Leese 2001), porcine (Booth et al. 2005) and bovine (Amin et al. 2014). Moreover, oxygen concentrations, either hypoxic or hyperoxic may increase ROS levels (Sena and Chandel 2012).
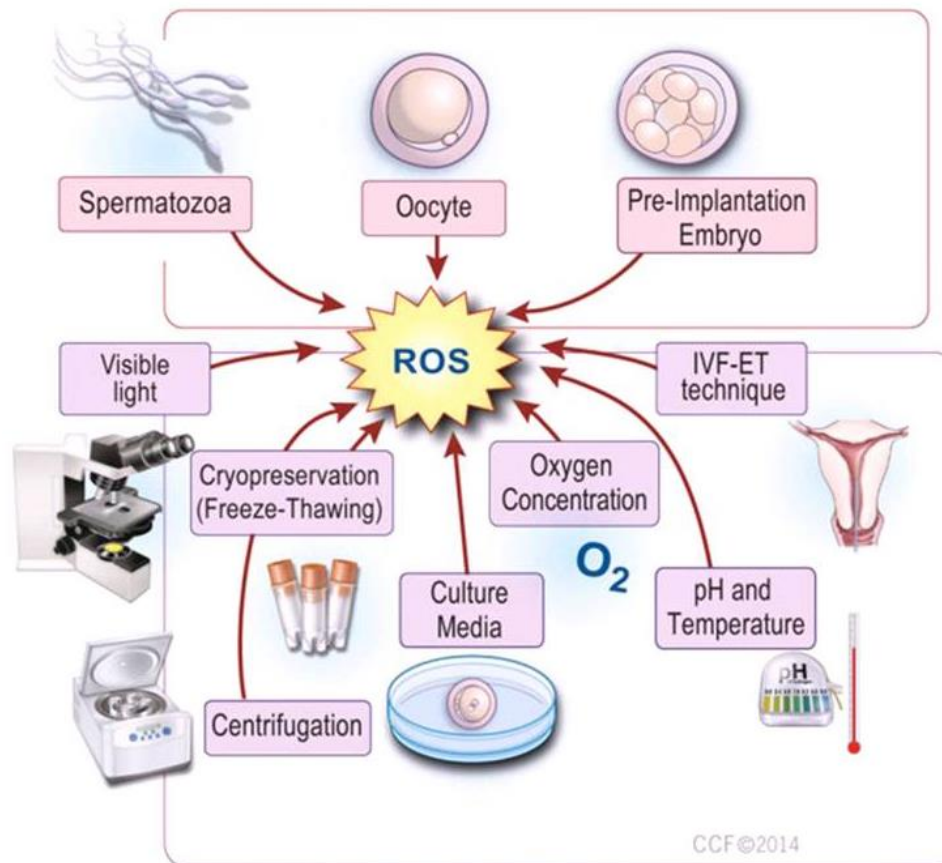
Figure 1.3. Exogenous sources of ROS affecting preimplantation embryos during in vitro production system. Adapter from (Agarwal et al. 2014)

In addition, visible light caused oxidative damage to unsaturated lipids within cell membrane as a result of photodynamic stress (Girotti 2001). Transient exposure to light could affect the embryo quality (Umaoka et al. 1992; Goto et al. 1993; Squirrell et al. 1999). In addition, cell culture media and additive can generate ROS depending on their composition and subsequently affect the quality of the oocyte maturation and the embryo development and quality (Agarwal et al. 2006). Serum is a commonly used additives in the media due to its composition (in terms of lipids, proteins, amino acids, growth factors, etc.), which accelerated the embryo development and resulting in early blastocyst formation under in vitro culture conditions (Enright et al. 2000; Rizos et al. 2002; Viuff et al. 1999). Moreover, serum albumin exhibits a powerful antioxidant activity (Alvarez and Storey 1983). On the other hand, serum contains high level of amine oxidase which considered a source of H_2O_2 production (Shannon 1978). Furthermore, ROS may be generated in spermatozoa mainly via the nicotinamide adenine dinucleotide-dependent oxido-reductase reaction at the mitochondrial level, which is considered a main source of

energy required for their motility (Henkel 2011). Spermatozoa act as carriers of ROS since they lack any scavenging system (Iwasaki and Gagnon 1992). The spermatozoa affected by oxidative stress can not only affect the oocyte after penetration, but other spermatozoa that remain outside induce oxidative stress in the media (Agarwal et al. 2006). In fact, the oocyte has the capability to repair sperm DNA damage as a result of oxidative stress. Unless the oocyte repair machinery is capable of repairing the oxidative DNA damage, the embryo will fail to develop (Alvarez 2003). DNA damage is associated with poor fertilization and embryonic loss. Therefore, detection of apoptotic or damaged spermatozoa before IVF is critical (Said et al. 2006; Chen et al. 2013).

1.2.2.3 Effect of oxidative stress on embryo development

The production of ROS above physiological level results in the oxidation of biological molecules such as lipids, proteins and DNA, which can strongly affect cell viability and eventually leads to cell death (Dalle-Donne et al. 2006; Vizcaya-Ruiz et al. 2009). Effect of ROS on sperm DNA has been associated with poor IVF outcomes (Saleh et al. 2003; Agarwal and Said 2005). Similarly, poor embryo quality and raised level of apoptosis was documented in IVF using sperm with abnormal DNA (Agarwal and Said 2005; Aitken and Krausz 2001). ROS can accelerate apoptosis in the cell by direct DNA damage, furthermore alteration of DNA repair mechanisms along with the important check points in the cell cycle (Barzilai and Yamamoto 2004). Moreover, another study showed the correlation between differential growth patterns and ROS levels in human embryo. Increasing ROS level was correlated with embryonic fragmentation and lower cleavage rates in intracytoplasmic sperm injection. Moreover, later stages of embryo development, the morula and blastocyst show varied response due to generation of oxidative stress in the media as well as in the number of dividing cells (Bedaiwy et al. 2004). Analysis of the affected embryos demonstrated a reduction in glutathione content, accelerated apoptosis involving the Caspase 3 system and increased membrane lesions in the inner cell mass (ICM) (Feugang et al. 2003).

1.3 Cellular response against oxidative stress

ROS need not to be harmful for the maintenance of normal cellular functions as long as the redox homeostasis is regulated. Indeed, ROS are important signaling messengers for proliferation, differentiation, apoptosis and other critical events during development (Ye et al. 2015). Under normal physiological conditions, accumulation of ROS is controlled by variety of endogenous antioxidant defense systems, both enzymatic and non-enzymatic, which either prevent or scavenge ROS (Mittler 2002; Zhou et al. 2014). There are various redox sensitive signaling pathways that are involved in the cellular response against oxidative stress through the regulation of transcription factors including AP-1 (Pinkus et al. 1996), NF-$_k$B (Jung et al. 2008; Kamata et al. 2005; Pham et al. 2004), MAPK (Zhou et al. 2006; Son et al. 2011), PI3K/AKT (Wang et al. 2000; Huang et al. 2001), PKC (Majumder et al. 2001), HSF1 (Pirkkala et al. 2001) and Nrf2 (Niture et al. 2014) (Figure 1.4). Moreover, enzymatic antioxidants including the superoxide dismutase (SOD), catalase (CAT), GPx, glutathione S-transferase (GST) and peroxiredoxin (Prx) (Michiels et al. 1994; Hayes and McLellan 1999), and the non-enzymatic antioxidants, including vitamins C and E, carotenoids, flavonoids (Machlin and Bendich 1987) are among the ROS scavenging molecules. Moreover, low molecular weight co-factor or peptides, such as GSH, thioredoxins (Trx) and NADPH also play an important role in the antioxidant defense (Zhou et al. 2014).

Alternative culture conditions during in vitro embryo culture system resulted in higher intracellular ROS level and altered the cellular transcriptomic profiles mainly in the activity of their NRF2 signaling pathway (Gad et al. 2012; Amin et al. 2014). In line with this, previous studies demonstrated the emerging role of Nrf2/Keap1 signaling pathway under oxidative stress conditions in bovine preimplantation embryos (Amin et al. 2014) and bovine granulosa cells (Saeed-Zidane et al. 2017).
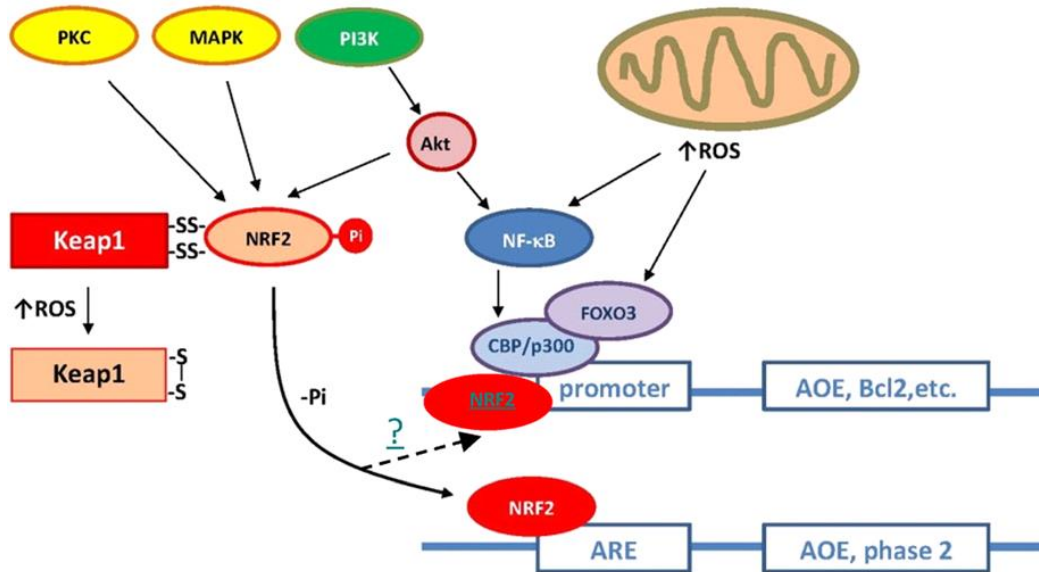
Figure 1.4. Signaling pathways involved in the cellular response against oxidative stress (Kurutas 2016).

1.4 Nrf2 regulating antioxidant defensive mechanisms

The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a master transcription factor involved in oxidative stress response (Moi et al. 1994). Nrf2 is a member of the cap 'n' collar (CNC) subfamily of basic region leucine zipper (bZIP) transcription factors enabling it to form heterodimer with ZIP domain of small musculoaponeurotic fibrosarcoma (Maf) proteins (Motohashi and Yamamoto 2004), resulted in induction of a variety of drug metabolizing enzymes (DMEs), such as glutathione S-transferase (GST) and NAD(P) H:quinone oxidoreductase 1 (NQO1) (Venugopal and Jaiswal 1996; Itoh et al. 1997).

1.4.1 Functional structure of Nrf2 and its activity

Nrf2 is a modular protein, which consists of 597 and 605 amino acids in mouse and human, respectively. The transcription factor contains seven highly conserved regions known as Nrf2-ECH homology (Neh) domains (Figure 1.5) (Itoh et al. 1995). The individual Neh domains have different functions. Neh1 contains the CNC–bZIP domain, which was first described as a DNA binding peptide sequence in Drosophila melanogaster (Mohler et al. 1991), and is essential for its transcriptional activity and mediates hetero-dimerization with Maf (Motohashi and Yamamoto 2004). The N-terminal Neh2 domain

negatively controls the activity of Nrf2, it interacts with a cytoplasmic protein Keap1 (Kelch-like ECH-associated protein 1) (Itoh et al. 1995), which interacts with two molecules of Keap1 via two binding sites, the high-affinity binding ETGE motif and the lower-affinity binding DLG motif (McMahon et al. 2004; Katoh et al. 2005). The Neh3–5 domains are involved in transcriptional activation of Nrf2 binding to various components of the transcriptional apparatus as the coactivator CREB binding protein (Katoh et al. 2001; Nioi et al. 2005). The Neh6 domain negatively controls Nrf2 (McMahon et al. 2004). It contains two conserved peptide motifs, DSGIS and DSAPGS, which are not recognized by Keap1, but are targeted for degradation by the E3 ubiquitin ligase β-TrCP. In addition, phosphorylation of Nrf2 by glycogen synthase kinase-3β enhances this alternative pathway, providing another mechanism for cellular control of Nrf2 activities (Rada et al. 2012; Chowdhry et al. 2013). Neh7 is the most recently described domain, which includes a region that is involved in a protein–protein interaction between Nrf2 and the DNA-binding domain of retinoid X receptor α (RXRα) to prevent recruitment of co-activators to the Neh4 and Neh5 domains leading to suppression of Nrf2 activity (Wang et al. 2013).
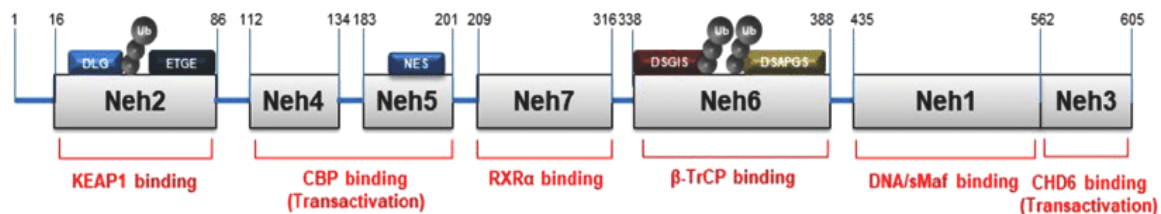
Figure 1.5. Structural function of nuclear factor E2-related factor 2 (NRF2) illustrated the domain structure of NRF2. Neh1 contains the CNC–bZIP domain, Neh2 domain negatively controls the activity of Nrf2, Neh3–5 domains are involved in transcriptional activation of Nrf2, Neh6 domain negatively controls Nrf2 and Neh7 involved in a protein–protein interaction. Adapted from Jung et al. (2018).

Nrf2 promoter analysis identified a DNA element that regulates basal expression and coordinated induction of genes encoding antioxidant enzymes in response to antioxidants and xenobiotics. This element was named ''antioxidant response element'' (ARE) because it was responsive to antioxidants (Dhakshinamoorthy et al. 2000). From studies over the past decade, the major emerging Nrf2 function is its role in oxidative stress resistance, which targeted genes, identified a number of ARE-containing genes involved in the control

of oxidant homeostasis (Zheng and Storz 2000). Nrf2 forms a heterodimer via leucine zipper with its obligatory binding partner small Maf proteins to be able to bind DNA (Itoh et al., 1997). Maf family divided into, small Maf proteins and large Mafs (v-Maf, c-Maf, Maf A, Maf B, and NRL) (Blank et al. 1997; Fujiwara et al. 1993). Moreover, Nrf2 has a competitor, Bach proteins which dimerize with small Maf (sMaf) proteins (Motohashi et al. 2002).

1.4.2 Regulation of Nrf2 activity

Under normal physiological conditions, Nrf2 is maintained at a low level due to its proteasomal degradation by ubiquitylation. This interaction is antagonized by oxidative stress enabling the CNC-bZIP protein to accumulate quickly and initiate a genetic program to allow cellular adaptation to stress (Itoh et al. 1999) (Figure 1.6). The prevailing view was that Nrf2 is mainly regulated at the protein stability level by Keap1, this somewhat myopic view because other studies reported that, β-TrCP, in conjunction with glycogen synthase kinase (GSK)-3, also represses Nrf2 (Salazar et al. 2006). In addition, protein stability play an important role in regulatory mechanism of Nrf2 activity can also be regulated by other transcription factors, post-translational modifications such as phosphorylation, acetylation and also other interacting protein partners which interfere with the formation of the ubiquitylation-competent Keap1–Nrf2 complex (Papp et al. 2012). The Nrf2 regulatory network in mice and human was summarized in several reviews discussing the latest findings regarding the epigenetic regulation of Nrf2 signaling by DNA methylation, histone modification, and microRNAs interactions (Hayes and Dinkova-Kostova 2014; Guo et al. 2015). Hence, it seems that in certain circumstances epigenetic and miRNA (miR)-based mechanisms are playing a role in prolonged, non-acute, changes in Nrf2 activity.
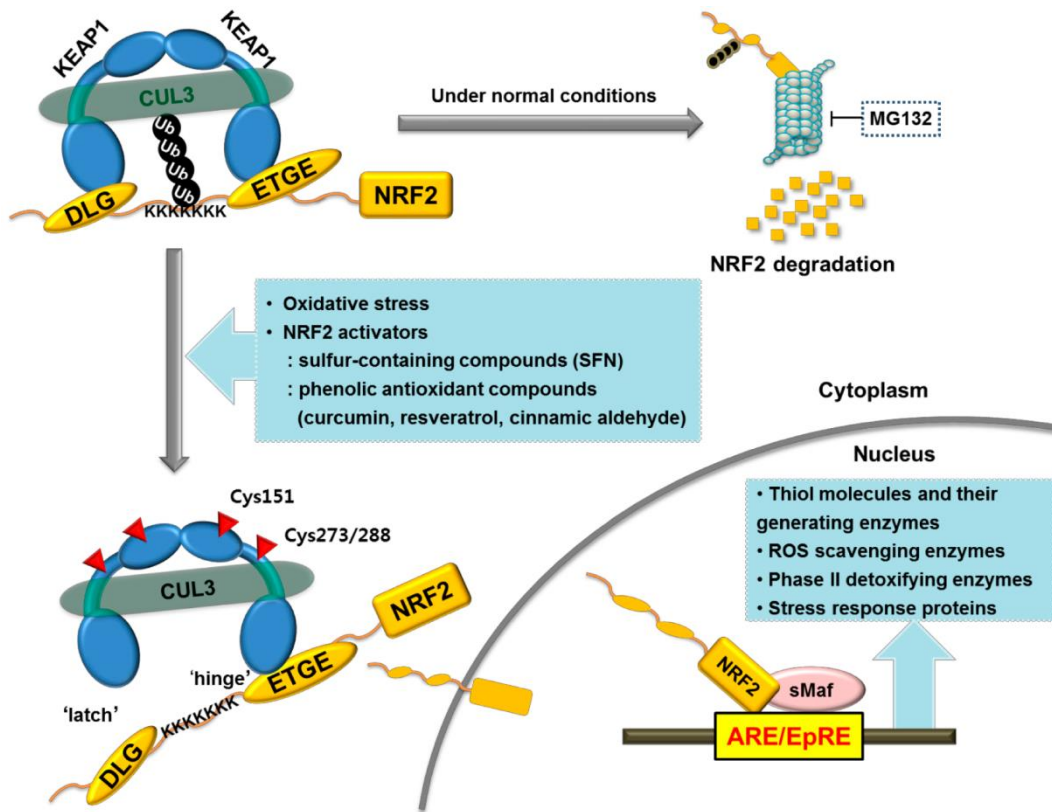
Figure 1.6. Nrf2/sMAF/ARE transcription pathway. Under normal conditions Nrf2 is stabilized through Keap-1 in the cytoplasm. Under oxidative stress Keap-1 are oxidized causing the dissociation of Nrf2, translocation to the nucleus, and binding to the antioxidant-responsive elements (ARE) and different antioxidant genes are induced. Adapted from (https://www.nwlifescience.com).

1.4.3 Epigenetic regulation of Nrf2

Epigenetic mechanisms play an important role in maintaining cellular differentiation (van Heesbeen et al. 2013). Moreover, epigenetic modifications are associated with dysregulation of multiple signaling pathways and alter gene expression without changing the gene sequence (Rodenhiser and Mann 2006; Migheli and Migliore 2012). Epigenetic regulatory mechanisms including DNA methylation, histone modification and miRNAs as shown in figure 1.7 (Puumala and Hoyme 2015).
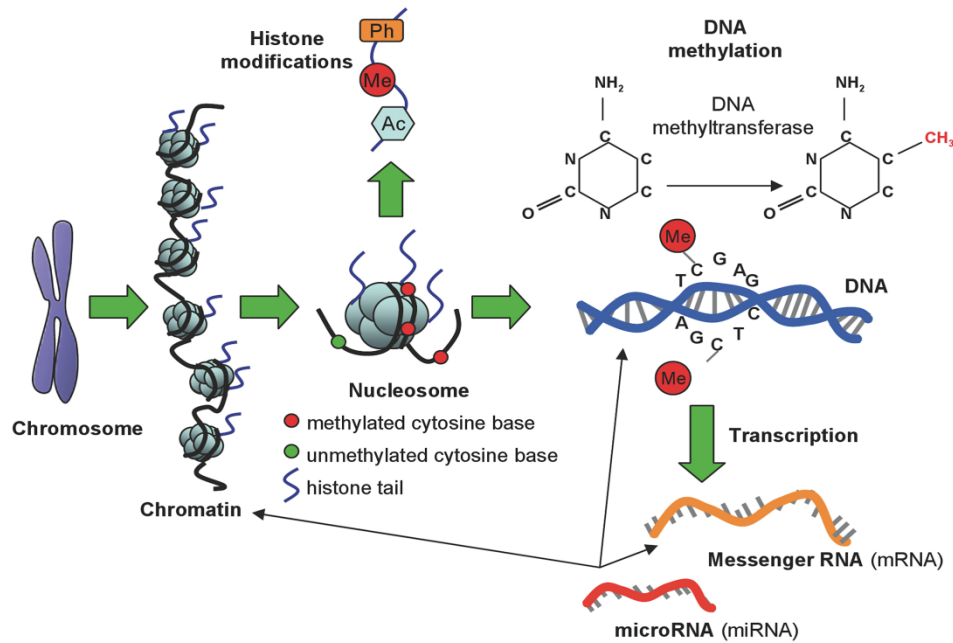
Figure 1.7. Epigenetic regulatory mechanisms including histone modification, DNA methylation and miRNAs interaction which involved in the regulation of gene expression on transcription or post-transcription level. Adapted from Gómez-Díaz et al. (2012).

DNA methylation is a well known epigenetic mechanisms that regulates gene expression (Bird 1986), and play a significant role in cellular development and differentiation (Laurent et al. 2010; Smith and Meissner 2013). DNA methylation developed as a result of addition of methyl group (CH3) to the cytosine nucleotide (C5) of cytosine residue in the position of CpG dinucleotide (CpGs) (Bird 1986; Jin et al. 2011), which is established and maintained during embryogenesis and regulated by a family of DNA methyltransferases (DNMTs). Dnmt3a and Dnmt3b are known as *de novo* DNMT, which establish a new methylation pattern to unmodified DNA. While DNMT1 maintain the DNA methylation pattern during DNA replication (Goto et al. 1994; Feng et al. 2005). Multiple studies demonstrated the correlation between the methylation patterns of CpGs within gene promoter region and gene expression pattern (Meissner et al. 2008; Lister et al. 2009; Varley et al. 2013), where hypermethylation was associated mostly with gene repression (Sproul et al. 2011; Sproul and Meehan 2013). Several cancer researches showed a connection between the tumors and down-regulation of Nrf2 and its downstream antioxidants in skin cancer mouse model (Xu et al. 2006), transgenic adenocarcinoma of mouse prostate (TRAMP) model (Yu et al. 2010) and human prostate cancers (Frohlich et

al. 2008). More importantly, CpGs were identified within the promoter region of Nrf2, which were hypermethylated in RAMP prostate tumors (Yu et al. 2010) and human prostate cancer samples (Khor et al. 2014). Interestingly, chemopreventive chemicals suppressed prostate tumerogenesis such as γ-tocopherol, showed repressing of hypermethylation of the Nrf2 promoter region and was associated with reactivation of Nrf2 and its downstream antioxidants (Huang et al. 2012).

Histone modifications are strongly correlated with the regulation of gene expression (Dong and Weng 2013). The DNA double helix is wrapped by four core histone proteins (octomers) to form repeating nucleosomes, which folded into chromatine fibers (Kornberg and Lorch 1999). Chromatin fibers have two structures, highly condensed low accessible chromatin with repressed gene transcription (heterochromatine) and loosely packed easily transcribed protein (euchromatin) (Huisinga et al. 2006). The posttranslational modifications occur in specific residues on the N-terminal tail of histones and play a crucial role in alteration of chromatin structure and subsequently the transcriptional activity of genes. Histone modifications include histone acetylation and methylation. Histone acetylation leads to neutralize the positive charge and destabilize the nucleosome structure facilitating the gene transcription process (Ura et al. 1997). Histone acetylation controlled by histone acetyltransferases enzymes (HATs) and HDACs, which add and remove the acetyl group respectively (Ura et al. 1997). Several studies showed the involvement of histone acetylation in Nrf2 regulation. HDACs are reported to inhibit the ARE-dependent gene expression. Lipopolysaccharide (LPS) was reported to induce inflammatory response in microglia cells and NF-κB subunit p65 promoted the interaction of HDAC3 with MafK, which facilitates the binding of HDAC3 to the ARE. This correlates with histone hypoacetylation in the local chromosome and represses the transcription of ARE-dependent genes (Wang et al. 2012). Moreover, HDACs inhibitors showed increasing histone acetylation in H3 and H4 and activate Nrf2- mediated antioxidant response (Correa et al. 2011; Wang et al. 2012).

Histone methylation play a crucial role in gene regulation, which could occur on all basic residues including histidines, arginines and lysines and within lysine could be mono (me1), di (me2) or tri (me3) methylated sites (Greer and Shi 2012). Histone methylation can lead to activation or repression of gene transcription based on which residue is methylated and the degree of methylation (Greer and Shi 2012). H3K27, which is a histone mark

associated with repressing the gene transcription, was found to be trimethylated (H3K27me3) as a result of activation of zester homolog 2 (Ezh2). In lung cancer, Ezh2 downregulation resulted in reduction of H3K27me3 at the promoter region of Nrf2 accompanied with upregulation of Nrf2 expression (Li et al. 2014a).

Furthermore, various studies reported the involvement of miRNAs in "fine-tuning" regulation of Nrf2 (Figure 1.8), more than 85 miRNAs including miR-144, miR-93, miR-29b-1, miR-365-1/miR-193b cluster, miR-27-a and miR-142-5p were reported to target the 3´-UTR region of Nrf2 mRNA resulting in reduction of Nrf2 expression level in different cell lines (Papp et al. 2012; Guo et al. 2015; Ayers et al. 2015).
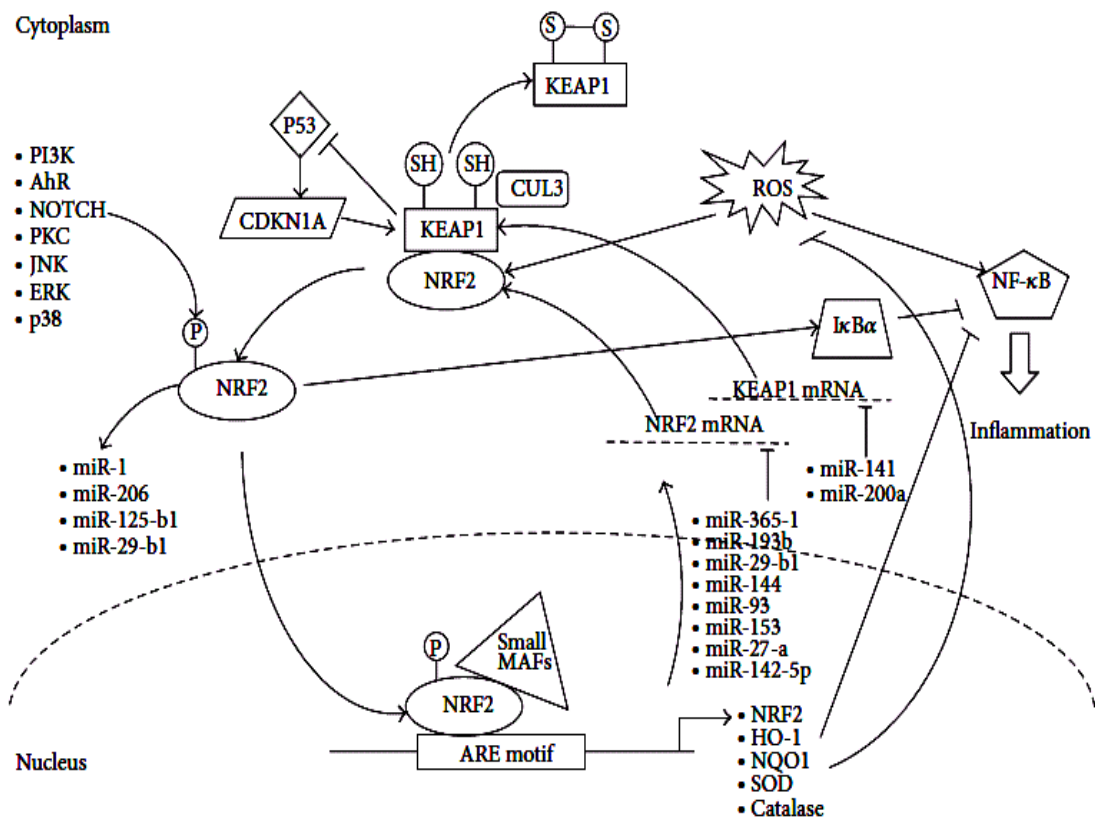
Figure 1.8. Nrf2 regulatory mechanism, highlighted miRNAs involved in the modulation of Nrf2 expression at transcriptional and\or translational level. Adapter from (Ayers et al. 2015)

1.5 miRNAs and genetic regulation

The non-coding RNAs (ncRNAs) are usually classified based on their nucleotide length into three groups: short ncRNAs, mid-size ncRNA and long ncRNA (Esteller 2011). Long ncRNAs (lncRNA) with nucleotide length more than 200 base pairs (bp) play a role in genomic imprinting, telomere regulation and X-chromosome inactivation (Geisler and Coller 2013). The mid-size ncRNAs including small nuclear RNAs with nucleotide length 60-300 bp act as a guide for ribosomal RNA modification (Deschamps-Francoeur et al. 2014). Short ncRNAs including miRNAs (miRNAs) 19-25 bp regulate gene expression (Mihailescu 2015) and piwi-interacting ncRNAs (piRNAs) with nucleotide length 26-31 bp which are involved in DNA methylation (Watanabe et al. 2011).

1.5.1 miRNA biogenesis and its regulation

MicroRNAs are endogenous, non-coding single stranded RNAs with the nucleotide length of approximately 19-25 bp found in plants and animals and are involved in posttranscriptional gene regulation (Bartel 2009). MicroRNA genes are transcribed by RNA polymerase II. Then, the biogenesis passes through two main processes in nucleus and cytoplasm. RNA polymerase III, Drosha and Dicer are responsible for transcription of miRNA genes. Within the nucleus, Drosha enzyme makes complexes with other protein containing dsRNA-binding domains (dsRBDs). In mammals, the Drosha partner is DiGeorge syndrome critical region gene 8 (DGCR8), this complexes processes the primary miRNAs (pri-miRNAs) to approximately 70 nucleotide precursor miRNAs (pre-miRNAs) (Du and Zamore 2005; Rana 2007). Then, pre-miRNAs are translocated to cytoplasm by Exportin 5 (Exp5), a Ran-GTP dependent transporter (Lee et al. 2003; Lund et al. 2004). Later, in cytoplasm, pre-miRNA is cleaved by another RNase III enzyme, Dicer. The Dicer enzyme forms complexes with TAR RNA-binding protein (TRBP) which cleaved to generate ~ 20 bp miRNA duplexes. One strand is functionally matured miRNA and its complimentary strand is degraded (Ketting et al. 2001; Hutvágner et al. 2001). The miRNAs are combined with ribonucleoprotein complexes (RNP) to form miRNA-induced silencing complex (miRISCs) and with the help of Argonaute proteins family (AGO1 to AGO4) to act as posttranscriptional regulators (Li and Rana 2012) (Figure 1.9). The miRNA binds to the 3′-untranslated region (UTR) of the target mRNA transcript with a 7– 8 nucleotide region called the 'seed sequence', resulting in blocking of translation through

RISC interaction with eIF6, which prevents assembly of 80S ribosomes, or through inhibition of translation after initiation

1.5.2 Regulatory role of miRNAs in the process of gametogenesis, endometrial receptivity, implantation and embryonic development

MicroRNAs have a regulatory role in female reproductive functions, in folliculogenesis, oocyte maturation, corpus luteum function, embryo implantation, and early embryonic development (Toloubeydokhti et al. 2008; Hossain et al. 2012; McGinnis et al. 2015) (Figure 1.9). Reproductive cycle is characterized by dynamic changes among cellular growth, angiogenesis, steroidogenesis and apoptosis, which are regulated at the endocrine and transcriptional levels. For that, understanding the molecular events, including the role of miRNA during the aforementioned events might provide a discernment view to enhance reproductive efficiencies.

Several studies demonstrate that Dicer1 knockdown female mice exhibited infertility and transplantation of wild type ovaries into a Dicer1 knockout females resulted in offspring, while transplant Dicer1 knockout ovaries to wild type females failed to establish pregnancies, which indicate that the fertility defect is correlated with the ovary (Otsuka et al. 2007, 2008). Moreover, Dicer1 knockdown mice exhibited normal ovulation, but the expression of LH receptor and prolactin receptor genes, which are necessary for corpus luteum formation/maintenance and subsequently progesterone level, were all decreased. Furthermore, recent studies on bovine granulosa cells showed differential expression patterns of miRNAs that could be associated with stage of follicular development and cell proliferation, differentiation, apoptosis and altered hormonal levels (Salilew-Wondim et al. 2014; Gebremedhn et al. 2015; Gebremedhn et al. 2016; Andreas et al. 2016).

Likewise, oviduct and uterus show drastically changes within the reproductive cycle, oviductal lumen surrounded by either secretory or ciliated cells. The activity of these cells is increased or decreased depending on estrogen and progesterone dominance, respectively. And also, uterus transition from highly proliferative non-receptive state to highly secretory receptive state when estrogen or progesterone is dominant, respectively (Ellington 1991; Hunter 2012; Besenfelder et al. 2012). Deletion of DICER 1 in female mice resulted in decrease the length and diameter of oviduct and uterus by more than half compared to the wild type counterparts (Nagaraja et al. 2008). Moreover, histological structure of uterus in

Dicer1 deleted mice showed a reduction in smooth muscle layer and uterine gland (Hong et al. 2008). In the same study they studied the effect of estrogen on miRNAs expression pattern, results showed that 30 miRNAs were upregulated and 19 miRNAs downregulated. Interestingly, miR-708 was one of the downregulated miRNAs that targets matrix metalloproteinase-9 (MMP-9), allowing MMP-9 translation, which is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development (Hong et al. 2008; Alexander et al. 1996; Anacker et al. 2011; Rana 2007; Plaks et al. 2013). In another study, the expression pattern of miRNAs in the mouse uterine site of embryo implantation and site of non-implantation sites showed that 13 miRNAs were upregulated and two were downregulated (Hu et al. 2008).

MicroRNAs play a vital role during oogenesis, spermatogenesis and early embryonic development. Several reviews are discussing the role of miRNAs during these events (Carletti and Christenson 2009; Maalouf et al. 2016; Luo et al. 2016; Hilz et al. 2016; Laurent 2008; Liu et al. 2016). Several studies revealed that, miRNAs are involved in activation and maintenance of primordial follicles (Zhang et al. 2013; Zhang et al. 2014), granulosa cell proliferation (Gebremedhn et al. 2016; Andreas et al. 2016; Pande et al. 2018), oocyte maturation and development (Pan et al. 2015; Sinha et al. 2017). During oocyte maturation and fertilization, Dicer1 expression pattern was strongly reduced specially at the two-cell stage embryo, which is accompanied by global degradation of maternal transcripts (Cui et al. 2007; Murchison et al. 2007). Previous studies demonstrate the impact of Dicer1 knockout on oocyte maturation, which is characterized by infertility, abnormal chromosomal alignment, and spindle disorganization leading to loss of meiotic maturation and polar body production (Murchison et al. 2007; Tang et al. 2007). Importantly, miRNAs expression pattern is altered depending on the follicular ovulatory status (Donadeu and Schauer 2013; Gebremedhn et al. 2015). Similarly, Dicer 1 knockout in mice testes resulted in alteration of miRNAs expression accompanied with spermatogenic failure and infertility (Greenlee et al. 2012). Moreover, environmental stressors lead to alteration of miRNAs expression during spermatogenesis (Rodgers et al. 2015; Grandjean et al. 2015). Zygotes developed from miRNA-depleted sperm revealed reduction in developmental potential (Yuan et al. 2016). Further, another study showed 14 miRNAs were expressed in sperm and 2-pronuclei embryos, but not in oocyte (Liu et al. 2012), suggesting the crucial role of parental miRNAs in embryo development. The

miRNA profile in mature oocyte and zygote is similar. However, after first division to 2-cell stage about 60% of miRNAs were downregulated (Tang et al. 2007). Moreover, Dicer1 and Dgcr8 knockouts were reported as embryonically lethal (Wang et al. 2007; Chen et al. 2012), which clarify that miRNAs are necessary for embryo development.
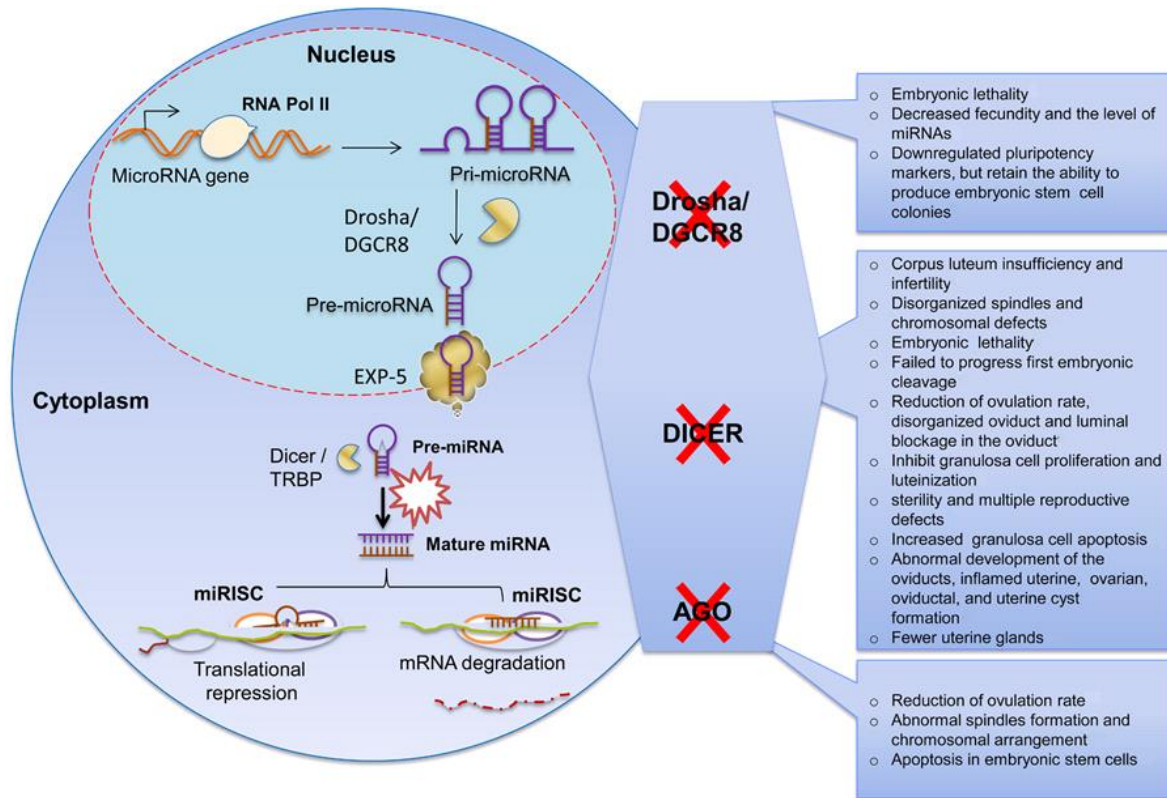
Figure 1.9. MicroRNA biogenesis and the impact of miRNA deficiency as a result of selective knockdown of Drosha/DGCR8, DICER or AGO, which affect on female reproduction and fertility. Modified figure from Tesfaye et al. (2018).

1.5.3 Oxidative stress altered miRNAs expression pattern

Recent studies demonstrated the crucial role of miRNAs in oxidative stress, inflammation and apoptosis (Berber et al. 2017). These miRNAs can target multiple oxidative stress-mediated pathways in female infertility disorders and can play an active role in maintaining redox homeostasis and cell viability, representing a new therapeutic solution. The correlation between miRNAs and oxidative stress was evaluated in several studies. H_2O_2-induced oxidative stress deregulated a total of 101 miRNAs including miR-708 in mouse hippocampal neurons, which are mainly involved in the regulation of MAPK pathway (Xu et al. 2012). In another microarray analysis, oxidative stress in diabetic

cardiomyocytes resulted in deregulation of 43 miRNAs, of which 37 miRNAs were downregulated and 6 miRNAs were upregulated out of total 351 miRNAs examined (Yildirim et al. 2013). The miRNAs expression is very sensitive to oxidative stress stimulation (Lin et al. 2009). Interestingly, oxidative stress induced by high H_2O_2 concentration mainly upregulated miRNAs expression. In contrary, lower H_2O_2 concentration dowenregulated the miRNAs expression (Wan et al. 2017).

1.6 Quercetin as potential antioxidants and Nrf2 activator

In order to counteract the oxidative damage of ROS, maintaining the physiological level of ROS through neutralization of the excessive ROS production, which could be achieved by supplementation of exogenous antioxidants leading to either prevent ROS formation or scavenging the existing ROS (Bansal and Bilaspuri 2010; Lampiao 2012). In the last decade, numerous studies focused on antioxidant supplementation to counteract the oxidative stress conditions and restore cellular antioxidant defense mechanisms. The Nrf2 signaling pathway reported as a common molecular target for numerous natural products. A variety of natural compounds was reported as activators of Nrf2 and exerts antioxidant activities including flavonoids, which are found among vegetables, fruits, nuts, seeds, stem, flowers, tea, and wine (Middleton and Kandaswami 1994). The flavonoids act through different modes including quenching free radical elements by donating a hydrogen atom or single-electron transfer, chelating metal such as Fe^{2+} and Cu^+, suppressing the enzymes associated with free radical generation such as xanthine oxidase, lipoxygenase, protein kinase C, cyclooxygenase, microsomal monooxygenase, mitochondrial succinoxidase, and NADPH oxidase, and stimulation of internal antioxidant enzymes (Procházková et al. 2011).

Flavonoids are natural phytochemical compounds, which exert antioxidant effects against oxidative stress conditions (Mierziak et al. 2014). Flavonoids antioxidant activities developed as a result of its chemical structures (Figure 1.10). The free hydroxyl groups (C in figure 1.10) will donate a hydrogen atom to radical molecules (Dugas et al. 2000). The hydroxyl structure in the B ring (Figure 1.10-a) by donating hydrogen and an electron to hydroxyl, peroxyl radicals (Santos and Mira 2004). The presence of C2-C3 unsaturated bond combined with C-4 carbonyl group in the C skeleton resulted in delocalization of electron from the B-ring (Figure 1.10-b; Wolfe and Liu 2008).
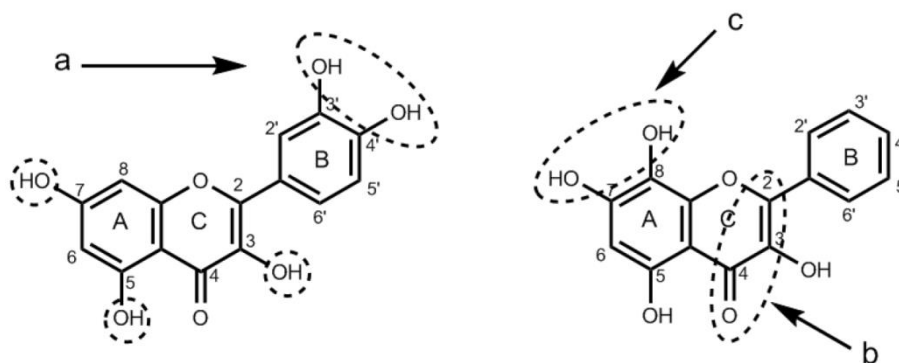
Figure 1.10. The antioxidant structure-activity relationship of flavonoids. Showing the ortho-dihydroxy arrangement in the B ring (a), the 2,3-double bond combined with the 4-keto groups in C ring (b) and hydroxyl groups in A ring (c). Adapted from Bubols et al. (2013).

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one) is a flavonoid (Figure 1.11), which exhibit a broad range of pharmaceutical properities including antioxidative effect (Morel et al. 1993; Vasilescu and Girma 2002; Ghosh et al. 2015), anti-inflammatory effect (Kang et al. 2013a; Saw et al. 2014; Sun et al. 2015) and antiapoptotic effect (Yang et al. 2014). Higher antioxidant activity was found to be correlated with the total number of hydroxyl group and mainly its presence within the B ring, which explain the potent antioxidant activity of quercetin compared to other compounds (Moalin et al. 2011). Based on the concentration, quercetin exerts the capacity to act either as a pro-oxidant or an antioxidant. High concentration of quercetin (40-100 µM) resulted in reduction of cell survival rate and downregulates the cellular antioxidant mechanisms. On the contrary, quercetin at low concentration (5-30 µM) upregulate the cellular antioxidant machinery (Robaszkiewicz et al. 2007; Samuel et al. 2012; Li et al. 2014b). Many studies reported the quercetin protect human RPE cells in vitro (Kook et al. 2008), porcine oocytes (Kang et al. 2013b), mouse embryos (Yu et al. 2014) and bovine embryos (Sovernigo et al. 2017) via the reduction of intracellular ROS level and activate antioxidant mechanisms. Moreover, it activates Nrf2 at transcriptional and translational levels (Tanigawa et al. 2007; Granado-Serrano et al. 2012).
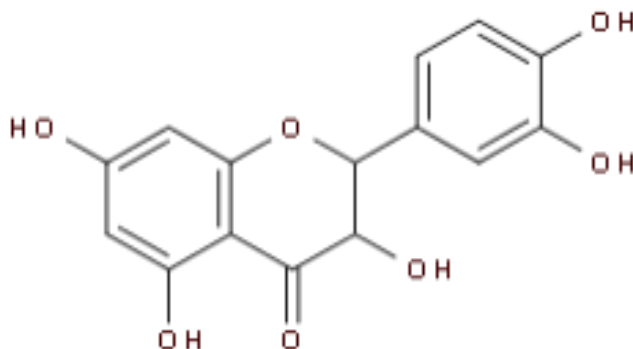
Figure 1.11. Chemical structure of quercetin. Adapted from (https://pubchem.ncbi.nlm.nih.gov)

1.7 Rationale and objectives of the study

Here, we hypothesized that miRNAs as an endogenous posttranscriptional gene modulators are involved in the Nrf2 regulatory in bovine granulosa cells exposed to oxidative stress conditions. Moreover, miRNAs mediate Nrf2 signaling pathway of preimplantation embryos cultured in media supplemented with quercetin.

For that, the present study aimed to investigate:

1. The regulatory role of microRNAs in the Nrf2 modulation under oxidative stress conditions or antioxidant supplementation (chapter 2)

2. The protective effect of quercetin against oxidative stress conditions in bovine granulosa cells and preimplantation embryos through Nrf2 activation (chapter 3).

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Chapter 2

Endogenous and Exogenous Modulation of Nrf2 Mediated Oxidative Stress Response in Bovine Granulosa Cells: Potential Implication for Ovarian Function

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

[1]Institute of Animal Science, Department of Animal Breeding and Husbandry, University of Bonn, Bonn, Germany

[2]Teaching and Research Station Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany

[3]Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

*Correspondence to:

Dr. Dawit Tesfaye, PhD

Department of Ainmal Breeding and Husbandry

Institute of Animal Science

Endenicher Allee 15,

Bonn 53115, Germany

E-mail: **tesfaye@itw.uni-bonn.de**

2.1 Abstract

Nrf2 is a redox sensitive transcription factor regulating the expression of antioxidant genes as defense mechanism against various stressors. The aim of this study is to investigate the potential role of noncoding miRNAs as endogenous and quercetin as exogenous regulators of Nrf2 pathway in bovine granulosa cells. For this cultured granulosa cells were used for modulation of miRNAs (miR-28, 153 and miR-708) targeting the bovine Nrf2 and supplementation of quercentin to investigate the regulatory mechanisms of the Nrf2 antioxidant system. Moreover, cultured cells were treated with hydrogen peroxide to induce oxidative stress in those cells. Our results showed that, oxidative stress activated the expression of Nrf2 as a defense mechanism, while suppressing the expression of those miRNAs. Overexpression of those miRNAs resulted in downregulation of Nrf2 expression resulted in higher ROS accumulation, reduced mitochondrial activity and cellular proliferation. Quercetin supplementation showed its protective role against oxidative stress induced by H_2O_2 by inducing the expression of antioxidant enzymes. In conclusion, this study highlighted the involvement of miR-153, miR-28 and miR-708 in regulatory network of Nrf2 mediated antioxidant system in bovine granulosa cells function. Furthermore, quercetin at a low dose played a protective role in bovine granulosa cells against oxidative stress damage.

2.2 Introduction

In mammals, the female reproductive performance is controlled by complex regulatory mechanisms in ovarian tissues in order to develop a competent oocyte which can induce pregnancy following fertilization and early embryo development (Son et al. 2011). During the reproductive lifespan, a cow is exposed to various stresses, including metabolic and heat stress, which results in disturbance of the redox hemostasis and development of oxidative stress (Garratt et al. 2011; Celi and Gabai 2015; Metcalfe and Alonso-Alvarez 2010). Oxidative stress is a phenomenon when the amounts of reactive oxygen species (ROS) surpass the cellular ability to counteract via generating scavenging antioxidant response system (Persson et al. 2014). ROS are reactive chemical species containing various forms of oxygen such as superoxide anion ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) (Aruoma 1998). ROS affects cellular functions by promoting cellular proliferation, differentiation, autophagy and apoptosis (Valko et al. 2007; Trachootham et al. 2009). Various studies have revealed the crucial role of ROS as central elements in cell signaling (Khan and Wilson 1995; Finkel 1998) and gene expression (Allen and Tresini 2000). Furthermore, ROS play a crucial role in follicular development (Chaube et al. 2005), oocyte maturation (Combelles et al. 2009), fertilization and embryo development (Agarwal et al. 2005). However, over accumulation of ROS resulted in hazardous effect on cellular micro- and macromolecules such as DNA, proteins and lipids, which are associated with cellular death (Alvarez 2003). Thus, maintaining the ROS at the basal level is essential for normal cellular functions. Recent study on the outcome of polycystic ovary syndrome (PCOS) in human on oocyte quality reported that, higher intracellular ROS level in PCOS granulosa cells induced cellular apoptosis, which contributes to poor oocyte quality and reduced the positive pregnancy outcomes (Lai et al. 2017). Thus, maintaining the cellular homeostasis via maintaining the equilibrium between ROS and cellular antioxidant machinery is required for the oocyte development and competency (Liang et al. 2017).

The nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2) is a redox sensitive transcription factor that regulates various genes involved in a various cellular functions and protection against endogenous and environmental stressors (Chanas et al. 2002; Kwak et al. 2003; McMahon et al. 2001; Hayes and Dinkova-Kostova 2014). Under normal conditions, Nrf2 is kept at basal homeostatic level in cytosol by its inhibitory protein;

Keap1 (McMahon et al. 2003). Upon exposure to stressors´ stimuli, Nrf2 is dissociated from Keap1 and subsequently is translocated into the nucleus, binding to an antioxidant response element (ARE) located at upstream promoter region of its downstream antioxidant target genes such as superoxide dismutase (SOD1), NAD(P)H:quinone oxidoreductase (NQO1), Peroxiredoxin (PRDX1) and catalase (CAT) (Itoh et al. 1997; Kensler et al. 2007; Kalayarasan et al. 2009; Taguchi et al. 2011) and facilitate the scavenging of the excessive ROS. In our previous studies, we have demonstrated the emerging role of Nrf2/Keap1 signaling pathway under oxidative stress conditions in bovine preimplantation embryos (Amin et al. 2014) and granulosa cells (Saeed-Zidane et al. 2017). In addition to the Keap1-mediated posttranslational control of Nrf2, another levels of regulatory network of Nrf2 through transcriptional, translational and other posttranslational modifications are reported (Hayes and Dinkova-Kostova 2014). Recently, it was shown that the Nrf2 signaling pathway could be regulated epigenetically via DNA methylation, histone modifications and interactions of microRNAs (miRNAs) (Guo et al. 2015).

MicroRNAs are short non-coding RNAs of 18-24 nucleotides long that play crucial role in posttranscriptional endogenous gene regulators, by binding to the 3´-untranslated region (3´-UTR) of the target mRNA resulting in either degradation of the target mRNA and\or reduced translation (Bartel 2004, 2009). Moreover, miRNAs exhibit species and cell type specific expression patterns (Ludwig et al. 2016; Sauer et al. 2017; Mor et al. 2011; Preusse et al. 2016). Our previous studies showed the crucial role of miRNAs in several biological functional processes in bovine granulosa cells including proliferation, differentiation, stress response and apoptosis (Blenkiron and Miska 2007; Andreas et al. 2016; Gebremedhn et al. 2016; Pande et al. 2018). Other studies have also evidenced the involvement of miRNAs in the regulation of reproductive functions including folliculogenesis (Hossain et al. 2012; Lei et al. 2010; Bahrami et al. 2017), oocyte maturation (Gilchrist et al. 2016; Sinha et al. 2017), corpus luteum function (Maalouf et al. 2016; Jerome et al. 2017) and early embryonic development (García-López and del Mazo 2012; Yuan et al. 2016; Yang et al. 2016). Nrf2 found to be regulated by several miRNAs including miR-28 in human breast cancer cells MCF-7 and human embryonic kidney cells 293T (HEK293T) (Yang et al. 2011) and miR-153 in human breast cancer cell lines (Wang et al. 2016), neuroblastoma cells (Narasimhan et al. 2012, 2014) and primary hippocampal

neurons (Ji et al. 2017). However, the role of miRNAs especially miR-28, miR-153 and miR-708 in regulation of Nrf2-mediated oxidative stress response mechanism in bovine granulosa cells and the subsequent cellular functions remain elusive.

Several attempts have been carried out to use antioxidant supplementation as a means of counteracting the oxidative stress conditions by restoring cellular antioxidant defense mechanism. For instance, flavonoids, which are natural phytochemical compounds exert antioxidant effects against oxidative stress conditions (Mierziak et al. 2014). Among flavonoids, quercetin (Que) (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one) considered the strongest antioxidant, which inhibit oxidant damage via different pathways (Morel et al. 1993; Vasilescu and Girma 2002; Ghosh et al. 2015). Previous studies showed that, Que has a broad range of pharmacological properties including antioxidant effect (Naderi et al. 2003; Kim et al. 2006; Bao et al. 2017; Yang et al. 2014), anti-inflammatory effect (Kang et al. 2013; Saw et al. 2014; Sun et al. 2015) and anti-apoptotic effects (Yang et al. 2014). Furthermore, Que is considered as a candidate chemo-preventive against oxidative stress in different human cell types (Chun et al. 2014). Previous studies indicated that, Que modulates several signaling pathways mainly the Nrf2 signaling pathway and interacts with cellular antioxidants defense system such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferase (GSTs), thioredoxin (TRX) and heme oxygenase 1 (HO-1) in rodents and human (Murakami et al. 2008; Ji et al. 2015; Ghanim et al. 2011). However, the exogenous regulation of Nrf2 signaling pathway via Que in bovine granulosa cells and subsequent cellular functions is still unknown.

Therefore, our study was designed to investigate the endogenous regulatory mechanisms of the Nrf2 signaling pathway via miRNAs and exogenous factor Que in bovine granulosa cells functions. Here, we specifically focused on the potential involvement of candidate miRNAs namely; miR-153, miR-28 and miR-708, which are predicted to regulate the Nrf2 gene by binding to the 3´-UTR of the mRNA, in regulating the signaling pathway under oxidative stress conditions. Furthermore, we aimed to investigate exogenous modulation of Nrf2 by supplementation of Que via either directly modulating the Nrf2 activity or by modulating the expression of the aforementioned candidate miRNAs and its ability to counteract hazardous effects of oxidative stress conditions in bovine granulosa cells.

2.3. Results

2.3.1. Nrf2 is targeted by miR-153, miR-28 and miR-708

In-silico analysis indicated the conserved binding site of miR-153, miR-28 and miR-708 are located between 105-112 and 58-65 of bovine Nrf2 3´-UTR (NM_001011678) (Figure 2.1A). The luciferase firefly activity was significantly reduced in cells co-transfected with the wild type plasmid constructs and bta-miR-153, miR-28 or miR-708 mimics compared to mutant and negative control plasmids co-transfected with miRNA mimics (Figure 2.1B).

2.3.2. Oxidative stress condition induced the expression of Nrf2 and suppressed the expression of miR-153, miR-28 and miR-708 in bovine granulosa cells

The effect of oxidative stress on the expression of Nrf2 and its associated candidate miRNAs was investigated in granulosa cells. Results showed that H_2O_2 treatment activated the expression of Nrf2 (Figure 2.1C), while the expression of miRNAs targeting Nrf2 namely; miR-153, miR-28 and miR-708 was reduced significantly (Figure 2.1D).

2.3.3. Overexpression of miR-153, miR-28 and miR-708 suppressed the expression of Nrf2 and its downstream antioxidants in bovine granulosa cells

Transfection of granulosa cells with the mimics of the candidate miRNAs has led to significant reduction in the expression of Nrf2 and its downstream antioxidant genes (NQO1 and PRDX1) (Figure 2.2A). Moreover, the protein abundance of Nrf2 was significantly decreased in cells transfected with miR-153, miR-28 and miR-708 mimics compared to the negative controls (Figure 2.2B). However, no alteration in the mRNA and protein expression level of Nrf2 and its downstream target transcripts was observed in cells treated with miRNA inhibitors (Figure 2.S1).

A

NRF2 Transcript
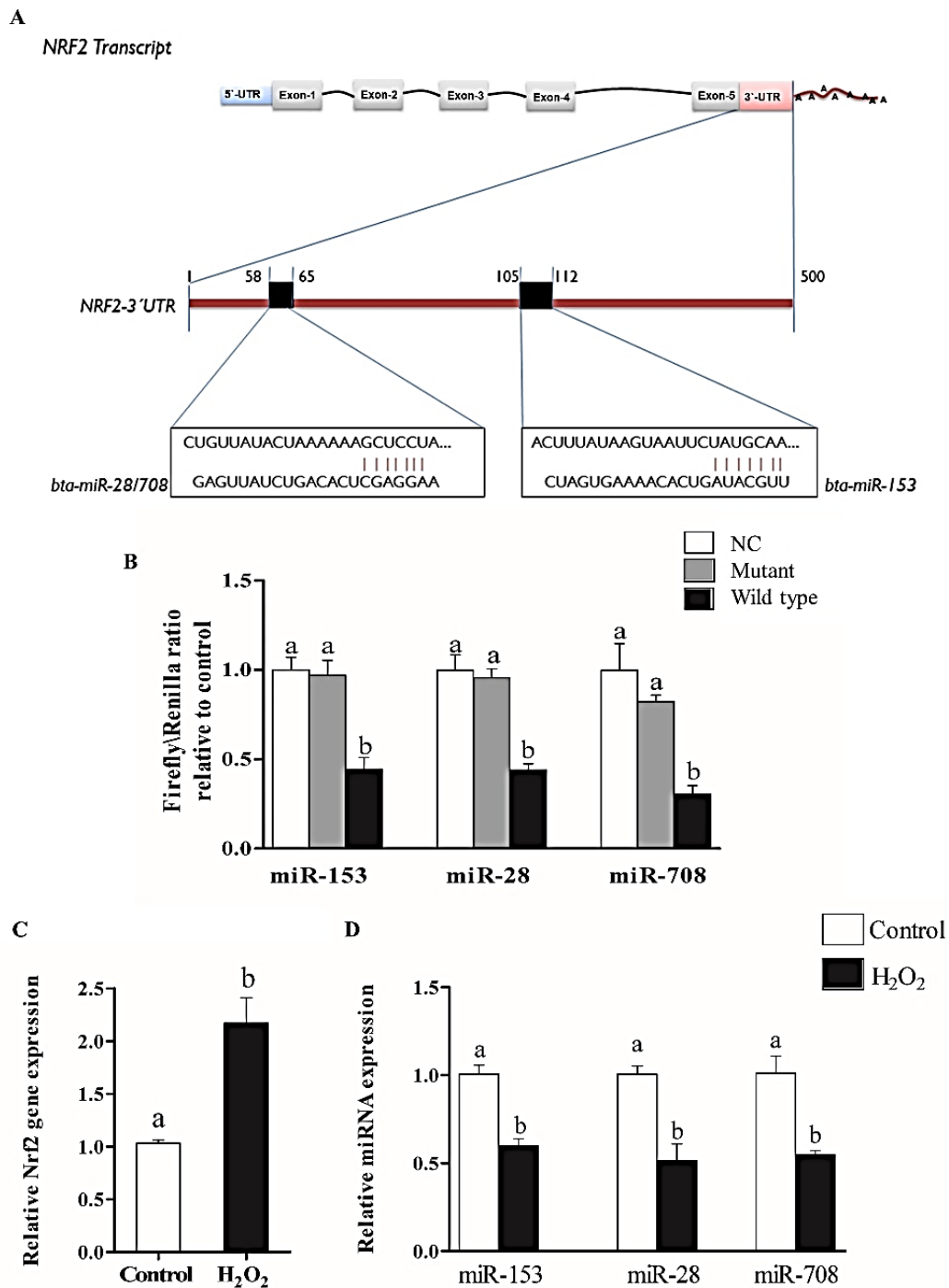
Figure 2.1. Conserved binding site of miR-153, miR-28 and miR-708 are located between 105-112 and 58-65, respectively in bovine Nrf2 3′-UTR (**A**). The luciferase firefly activity is reduced in granulosa cells co-transfected with bta-miR-153, miR-28 and miR-708 mimics with the wild type pmiRGlo expression vector (**B**). H_2O_2-induced oxidative stress increased cellular mRNA expression level of Nrf2 (**C**), as well as decreased miR-153, miR-28 and miR-708 expression level (**D**). qRT-PCR analysis of Nrf2, miR-153, miR-28 and miR-708 in granulosa cells under normal condition (white bar) and oxidative stress condition (black bar). Data are presented mean ±SEM of three independent biological replicates. Bars with different letters (a,b) showed statistically significant differences ($p<0.05$).
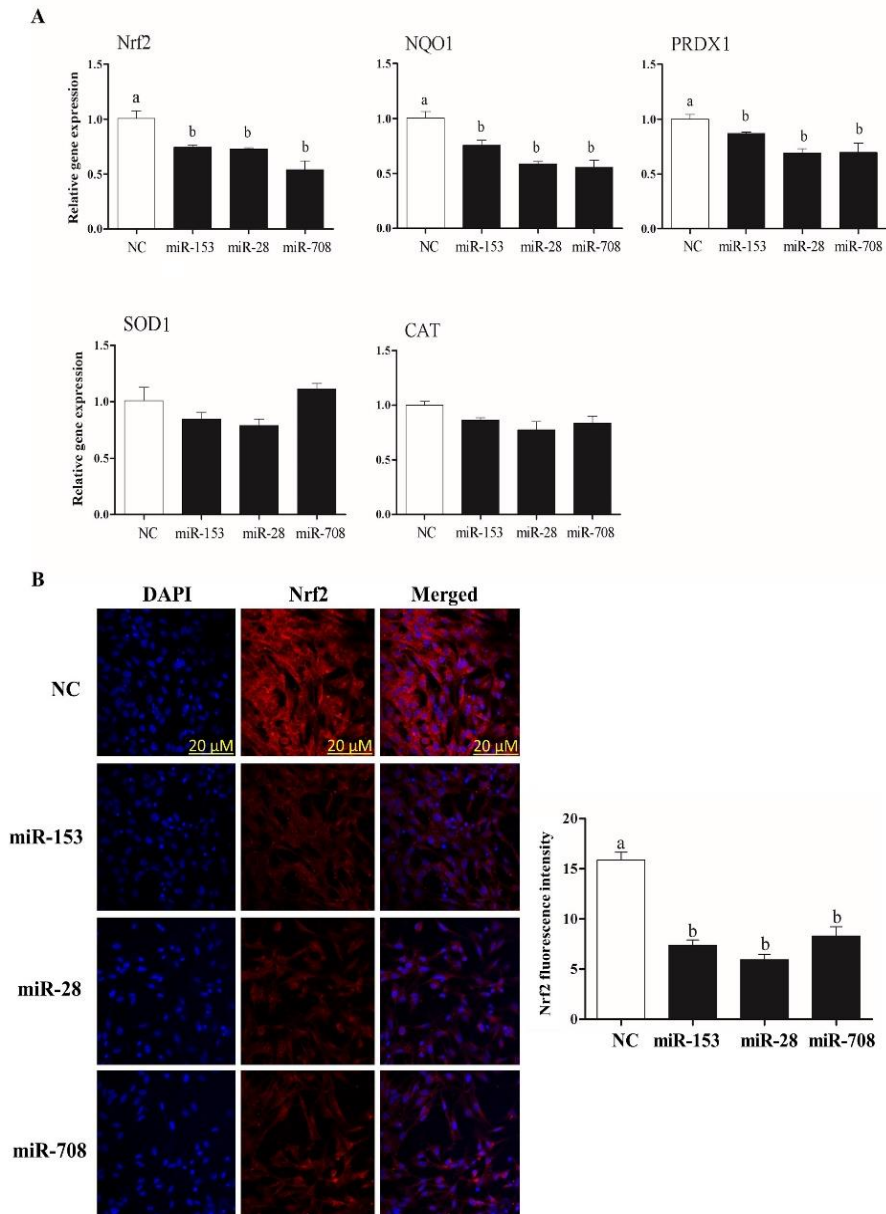
Figure 2.2. Quantitative RT-PCR of Nrf2 and its downstream antioxidants in bovine granulosa cells co-transfected with miR-153, miR-28 and miR-708 mimics (**A**). Immunocytochemistry of Nrf2 in bovine granulosa cells co-transfected with miR-153, miR-28 and miR-708 mimics (**B**). Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b) showed statistically significant differences (p<0.05).

2.3.4. Overexpression of miR-153 or miR-28 and miR-708 increased intracellular ROS level, reduced mitochondrial activity and cell proliferation rate in bovine granulosa cells

To determine whether deregulation of Nrf2 levels due to overexpression of miR-153, miR-28 and miR-708 is accompanied by the corresponding oxidative stress phenotypes, the intracellular ROS, mitochondrial activity and cellular proliferation were determined following overexpression of the candidate miRNAs. Results revealed that reduction of Nrf2 expression via miR-153, miR-28 and miR-708 resulted in a significant increase in intracellular ROS compared to the negative control, accompanied by lower mitochondrial activity (Figure 2.3A), and reduced the rate of granulosa cell proliferation (Figure 2.3B).

2.3.5. Selective knockdown of bovine Nrf2 impaired bovine granulosa cell functions

In order to cross-validate the regulatory role of miR-153, miR-28 and miR-708 in Nrf2 suppression, the Nrf2 expression was selectively suppressed using siRNA. Cells co-transfected with siRNA-Nrf2 showed a significant reduction of Nrf2 mRNA and protein level compared to negative control (Figure 2.4A and C).

The reduction of Nrf2 expression level resulted in reduced the expression of downstream antioxidant genes (Figure 2.4A), and cell proliferation rate (Figure 2.4B), increased intracellular ROS level and reduced mitochondrial activity (Figure 2.4D)

A
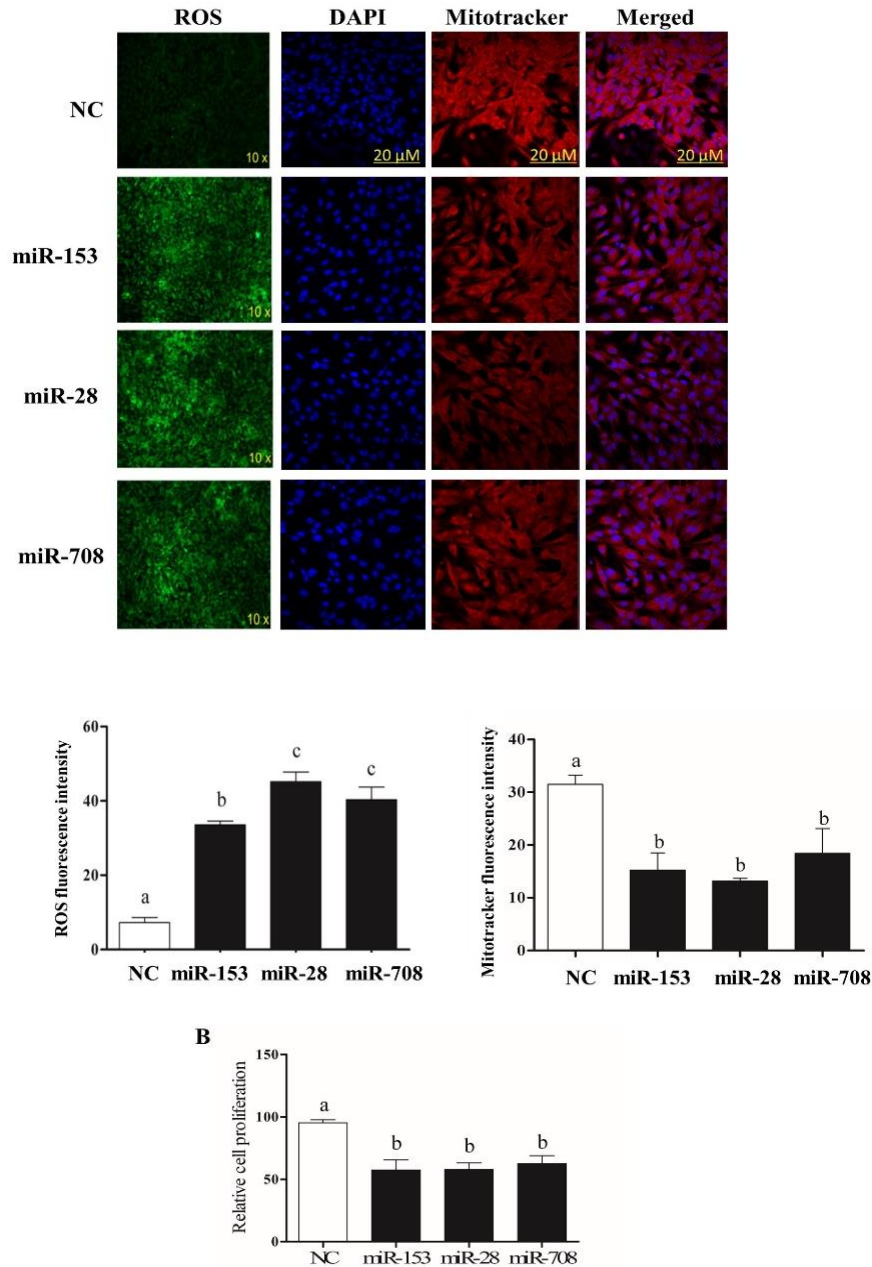
Figure 2.3. Higher level of intracellular ROS level accompanied with lower mitochondrial activity in bovine granulosa cells co-transfected with miR-153, miR-28 and miR-708 (**A**). Moreover, overexpression of miR-153, miR-28 and miR-708 resulted in reduced bovine granulosa cell proliferation (**B**). White bar indicates the proliferation rate of the negative control and black bars represent proliferation rate of cells co-transfected with miRNA mimics. Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b) showed statistically significant differences (p<0.05).
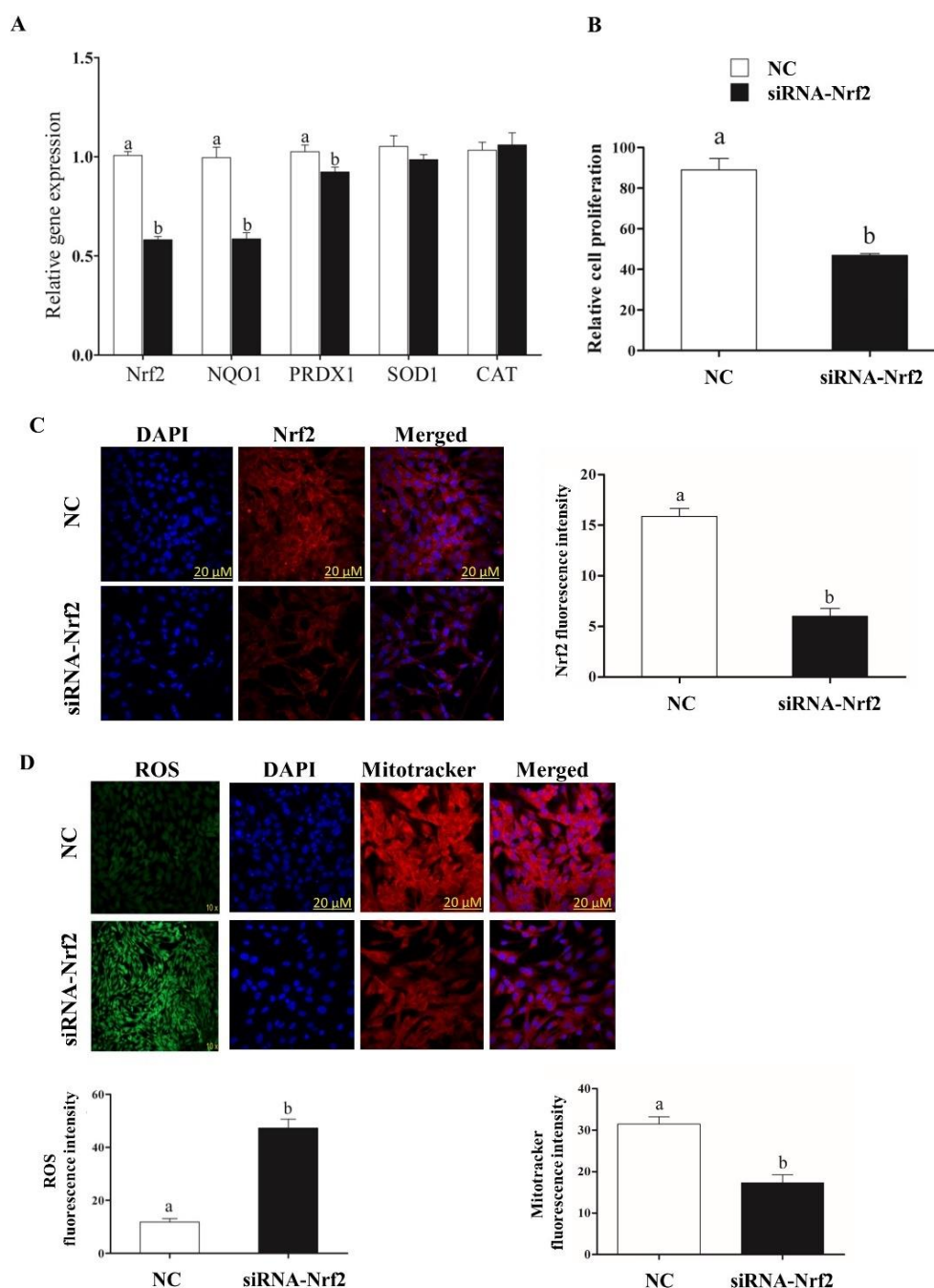
Figure 2.4. Selective knockdown of Nrf2 reduced cellular mRNA expression level of Nrf2 and its downstream antioxidant genes (**A**). Selective knockdown of Nrf2 reduced bovine granulosa cell proliferation (**B**). Immunocytochemistry of Nrf2 in bovine granulosa cells co-transfected with siRNA targeting the Nrf2 (**C**). Moreover, higher intracellular ROS level and reduced mitochondrial activity (**D**) was observed in Nrf2-knockdown cells compared to the control counterparts.. White bars indicate the cells transfected with the NC and the dark bars indicate cells transfected with siRNA targeting Nrf2. Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b) showed statistically significant differences ($p<0.05$).
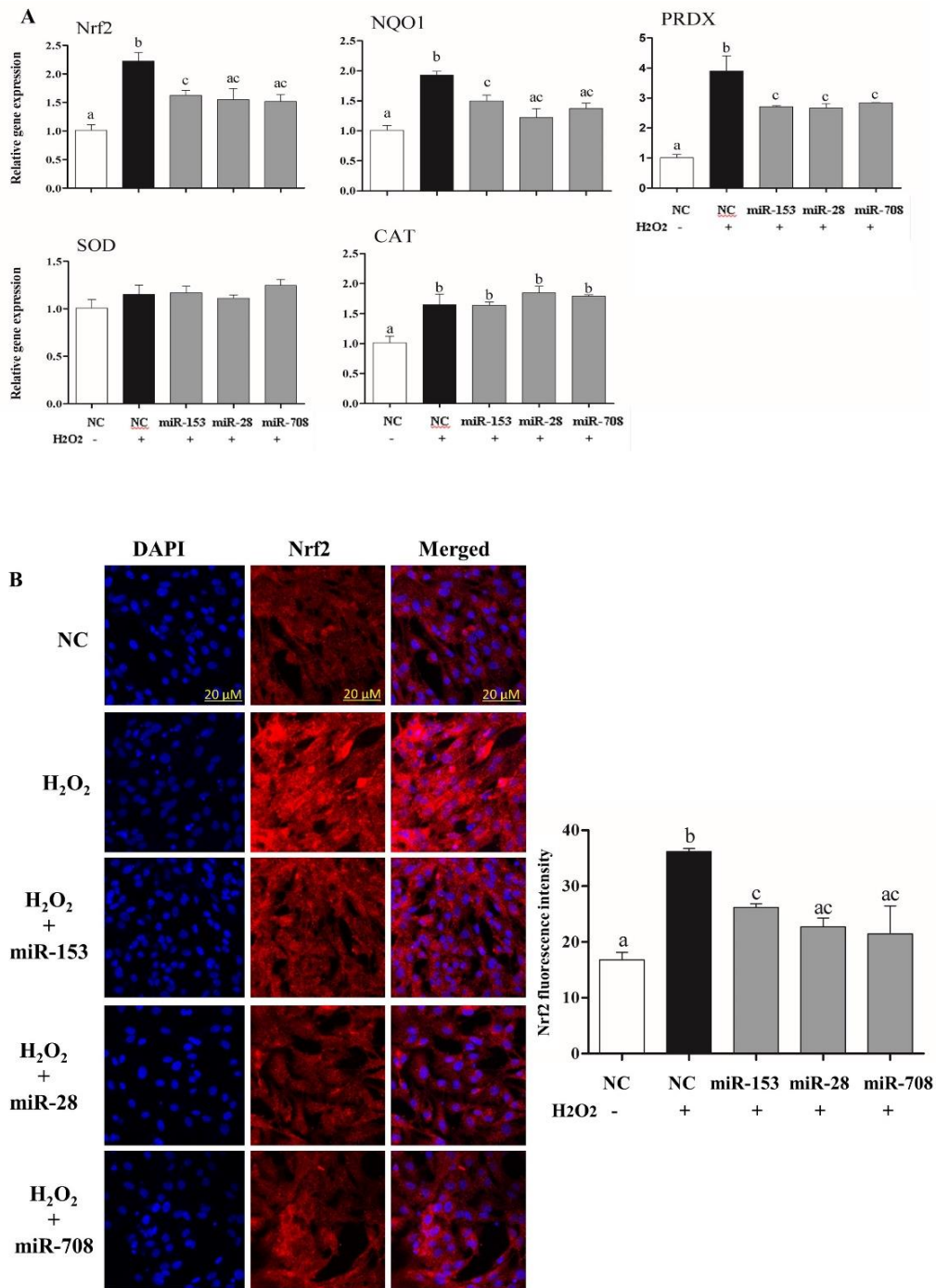
Figure 2.5. Overexpression of miR-153, miR-28 and miR-708 under oxidative stress conditions reduced mRNA expression level of Nrf2 and its downstream antioxidants (**A**)..Immunocytochemistry of Nrf2 in bovine granulosa cells co-transfected with miR-153, miR-28 and miR-708 mimics under oxidative stress conditions. The Nrf2 protein expression level was reduced under oxidative stress conditions followed by transfection with miR-153, miR-28 and miR-708 mimics individually compared to H_2O_2- treated group (**B**). White bars indicate the cells transfected with negative control without H_2O_2. Dark bars indicate cells transfected with negative control under H_2O_2 and gray bars represent cells transfected with miRNA mimics under the H_2O_2 challenge. Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b,c) showed statistically significant differences ($p<0.05$).

2.3.6. Overexpression of miR-153, miR-28 and miR-708 under oxidative stress negatively impact on bovine granulosa cell functions

The consequence of modulation of Nrf2 targeting miRNAs for cells defense mechanisms was investigated under oxidative stress condition. As expected, overexpression of miRNAs targeting the Nrf2 gene decreased both the mRNA and protein levels of Nrf2 and its downstream antioxidants (Figure 2.5A and B). Moreover, overexpression of the candidate miRNAs resulted in induction of ROS and reduction of mitochondrial activity (Figure 2.6A) and reduced cellular proliferation rate (Figure 2.6B)

2.3.7. Quercetin enhanced bovine granulosa cell functions under oxidative stress conditions by inducing the Nrf2 expression and its downstream antioxidants

Treatment of cultured granulosa cells with Que resulted in a significant increment in the expression of Nrf2 and its downstream antioxidant genes in dose dependent manner (Figure 2.S2A). Moreover, supplementation Que at a dose of 10 µM showed significant increase on the expression of Nrf2 protein (Figure 2.S2B), slight reduction on intracellular ROS level (Figure 2.S3A), increasing mitochondrial activity (Figure 2.S3B), slightly enhanced the cellular proliferation rate and the cell cycle (Figure 2.S4A and B). Thus, Que at a dose of 10 µM was selected for further analysis under oxidative stress condition.

To assess the rescuing role of Que, bovine granulosa cells exposed to oxidative stress for 40 min were then treated with 10 µM Que. Results showed that treatment of cells with Que resulted in upregulation of Nrf2 (Figure 2.7A and B), accompanied by increased cellular proliferation rate (Figure 2.8A), reduced intracellular ROS level (Figure 2.8B) and increased mitochondrial activity (Figure 2.8C) in H_2O_2-Que treated cells compared to H_2O_2 treated alone, suggesting the rescuing effect of Que against oxidative stress condition through activation of Nrf2 signaling pathway.

Furthermore, the induced expression of Nrf2 gene by Que in cultured granulosa cells was accompanied by concomitant reduction in the expression of miR-153, miR-28 and miR-708 (Figure 2.9).
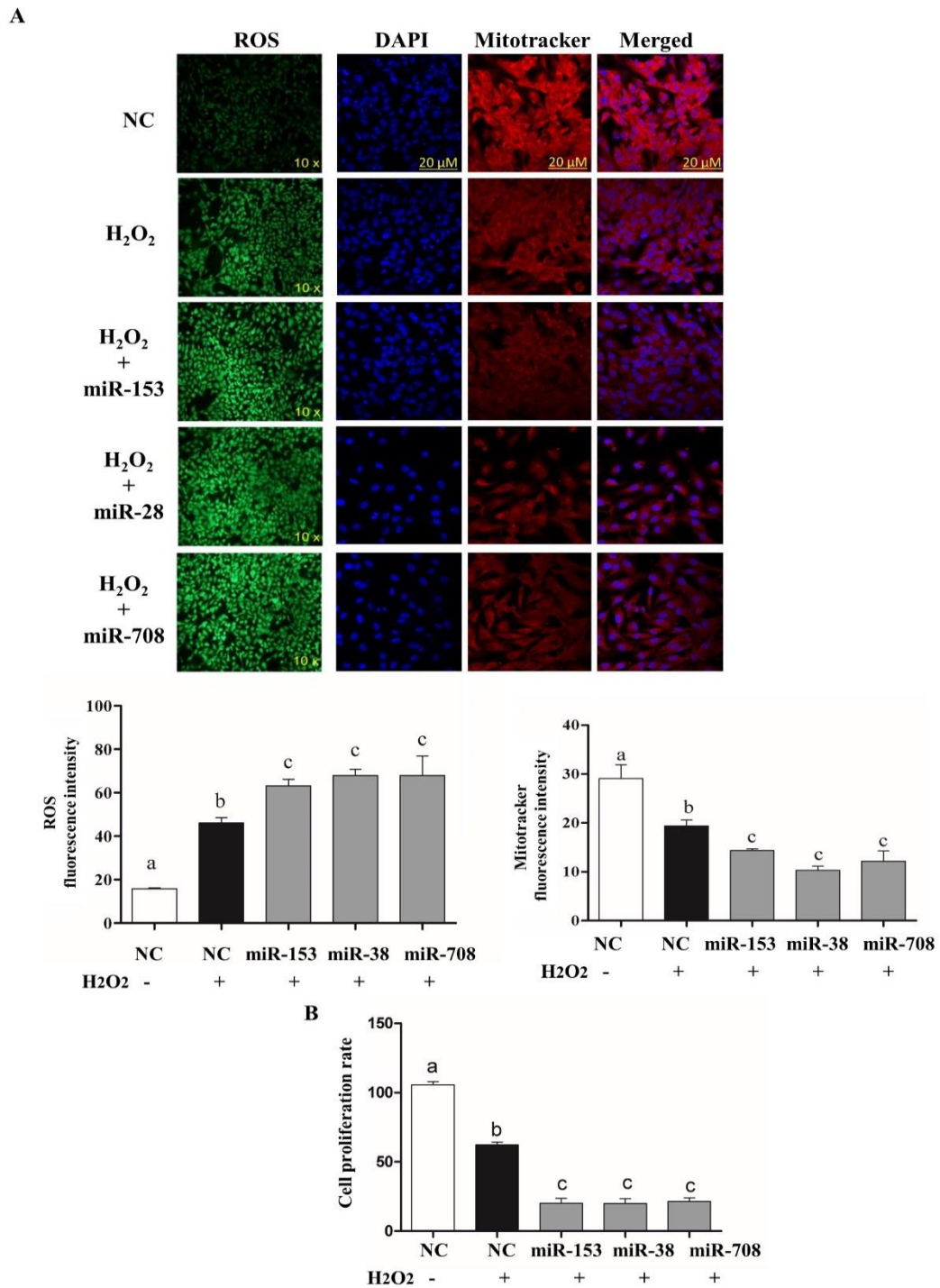
Figure 2.6. Overexpression of miR-153, miR-28 and miR-708 under oxidative stress conditions increased the intracellular ROS level and reduced the mitochondrial activity compared to cells treated with only H_2O_2 (**A**). Moreover, bovine granulosa cell proliferation was significantly reduced in cells transfected with miRNA mimics under H_2O_2 (**B**). White bars indicate the cells transfected with negative control without H_2O_2. Dark bars indicate cells transfected with negative control under H_2O_2 and gray bars represent cells transfected with miRNA mimics under the H_2O_2 challenge. Data are presented as mean $\pm$SEM of three independent biological replicates. Bars with different letters (a,b,c) showed statistically significant differences (p<0.05).

A
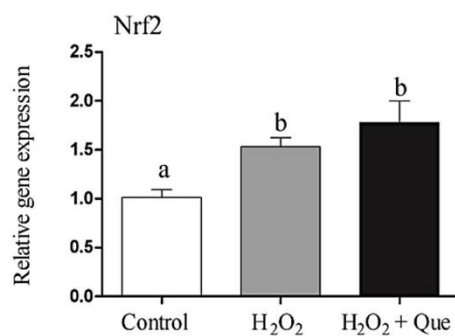
B
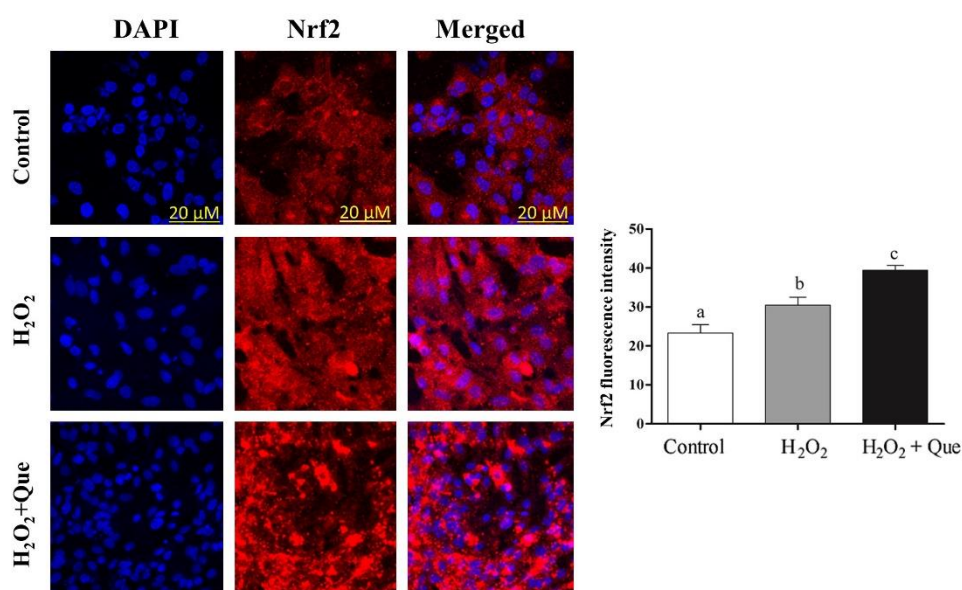
Figure 2.7. Treatment of cells with quercetin showed slight increment in the expression of Nrf2 under oxidative stress (A). Bovine granulosa cells co-incubated with quercetin under oxidative stress condition showed upregulation of Nrf2 at protein level (B). The white bar indicates the control group and the gray and black bars indicate H_2O_2 treated and cells treated with both H_2O_2 and quercetin, respectively. Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b,c) showed statistically significant differences ($p < 0.05$).
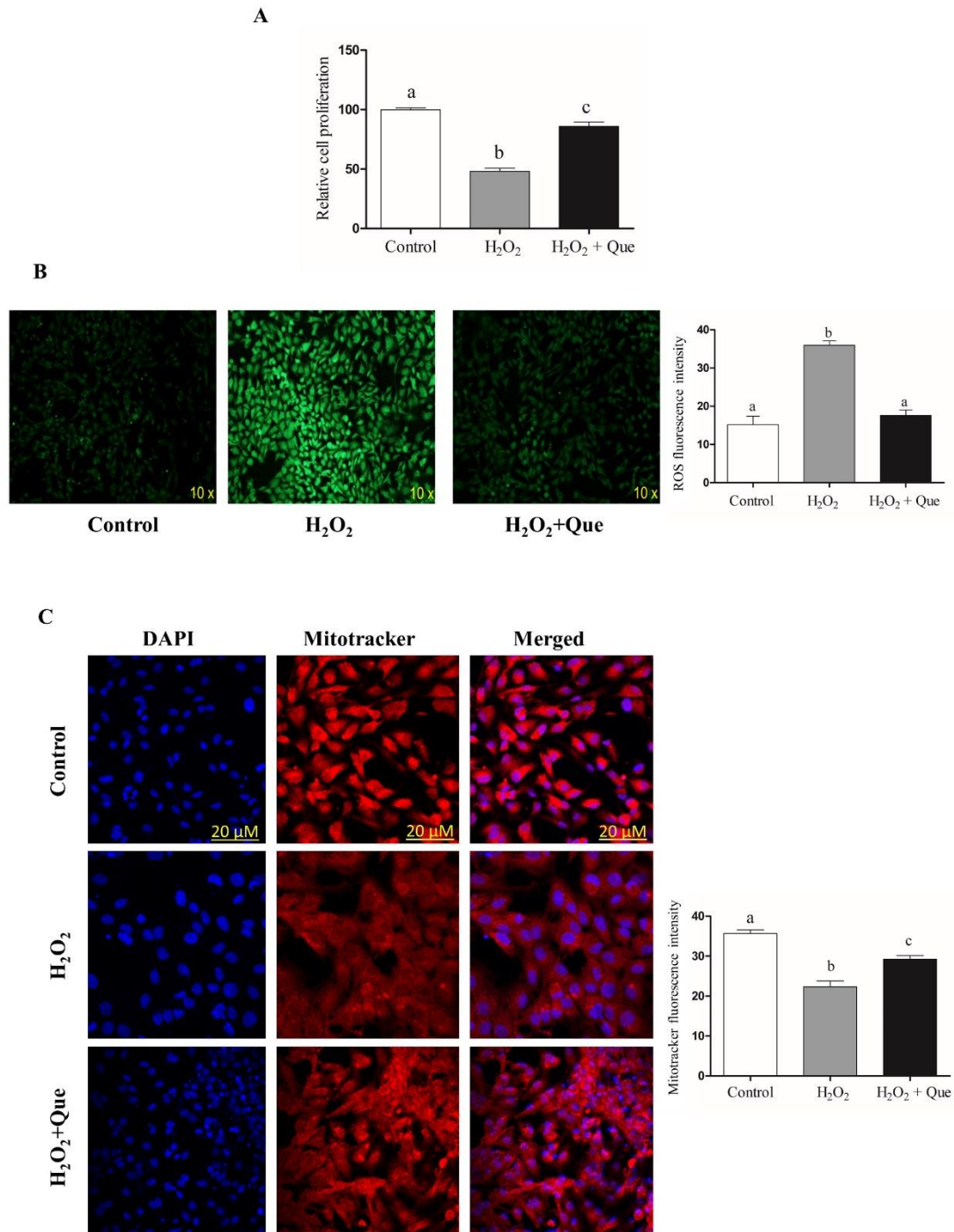
Figure 2.8. Quercetin rescue bovine granulosa cells from the oxidative stress damage induced by H_2O_2. Supplementation of quercetin following H_2O_2 treatment increased granulosa cells proliferation (**A**), reduced intracellular ROS level (**B**) and increase mitochondrial activity (**C**) compared to cells treated with only H_2O_2. The white bar indicates the control group, while the gray and dark bars represent cells treated with only H_2O_2 and cells treated with both H_2O_2 and quercetin, respectively., Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b,c) showed statistically significant differences ($p<0.05$).
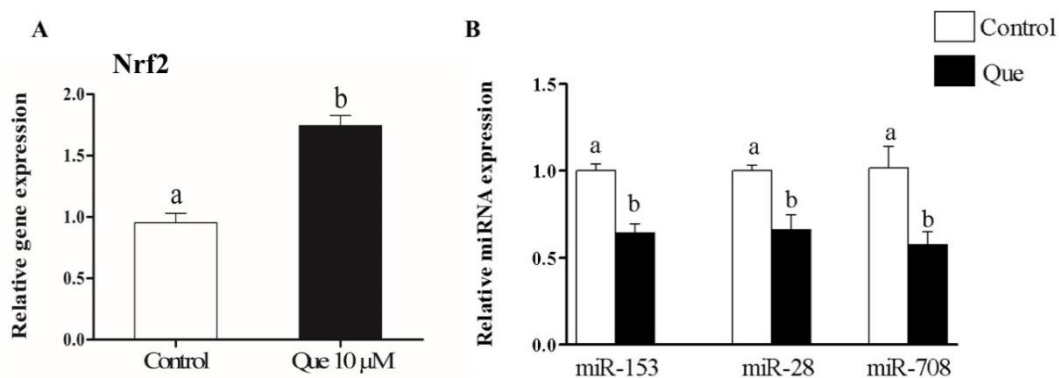
Figure 2.9. Quercetin increased cellular mRNA expression level of Nrf2 (**A**). The expression of miR-153, miR-28 and miR-708 was downregulated following quercetin treatment of cells(**B**). White bar represent the control groups and dark bars represent quercetin treated group. Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b) showed statistically significant differences (p<0.05).

2.4. Discussion

Mammalian ovary is a metabolically active organ, which generates excess amount of ROS during the final stages of follicular development and ovulation (Pandey et al. 2010; Shkolnik et al. 2011). ROS have both positive and deleterious effect in mammalian ovaries. Physiological level of ROS act as a signaling transducer required for growth factor signaling transduction and physiologic adaptation phenomena (Finkel 2011; Cordeiro et al. 2005; Betteridge 2000), oocyte maturation (Martín-Romero et al. 2008; Tripathi et al. 2009), ovarian steroid biosynthesis (Fujii et al. 2005) and intermediate decisive changes in cumulus cells prior to ovulation (Shkolnik et al. 2011). Moreover, oxidative stress conditions showed mitochondrial morphologic and degenerative changes in mouse granulosa cells. In addition, oxidative stress significantly reduced plasma progesterone and testosterone level, leading to fertility problems in mice (Shi et al. 2016). In our previous study, we showed the crucial role of Nrf2 and its downstream antioxidants for the survival of bovine granulosa cells (Saeed-Zidane et al. 2017) and preimplantation embryos (Amin et al. 2014) cultured in vitro under oxidative stress conditions. Others have evidenced the correlation between suppression of Nrf2 and inhibition of cellular proliferation (Fan et al. 2017; Homma et al. 2009), pronounced increase in ROS production (Duffy et al. 2015; Holmström et al. 2013) and lower mitochondrial activity (Holmström et al. 2013). Here we have evidenced the presence of several endogenous and exogenous regulatory mechanisms in cultured bovine granulosa cells exposed to oxidative stress.

Target gene prediction tools indicate that more than 85 miRNAs including, miR-144, miR-93, miR-29b-1, miR-153, miR-365-1/miR-193b cluster, miR-28, miR-27-a and miR-142-5p potentially target the Nrf2 gene (Guo et al. 2015; Ayers et al. 2015; Papp et al. 2012). Moreover, miR-28 and miR-153 were previously reported to be involved in Nrf2 regulatory network in breast cancer and neuroblastoma cells (Yang et al. 2011; Wang et al. 2016; Narasimhan et al. 2014; Narasimhan et al. 2012). On another hand, miRNAs known to be differentially expressed in cell type, tissue and species specific manner (Ludwig et al. 2016; Mor et al. 2011; Sauer et al. 2017; Preusse et al. 2016). Taking these findings into consideration and based on in silico analysis, miR-153, miR-28 and miR-708 were investigated for their role in regulating Nrf2 under oxidative stress conditions in bovine granulosa cells. Treatment of bovine granulosa cells with H_2O_2 increased expression of Nrf2 gene accompanied by reduction in expression of miR-153 and miR-28 and miR-708 (Figure 2.1). This is in agreement with previous studies showing an inverse relationship between Nrf2 expression pattern and miR-28 in breast carcinoma cell lines (Yang et al. 2011), miR-93 within rat models of breast carcinogenesis (Singh et al. 2013) and miR-153 in breast cancer cell lines (Wang et al. 2016), neuroblastoma cells (Narasimhan et al. 2012; Narasimhan et al. 2014) and glioma stem cells (Yang et al. 2015). Modulation of miR-153, miR-28 and miR-708 expression individually resulted in reduction of the expression of Nrf2 both at mRNA and protein level with concomitant reduction in expression its downstream antioxidant genes. The negative interaction between the candidate miRNAs and the Nrf2 gene was validated by the selective knockdown of the Nrf2 gene using siRNA designed to target the Nrf2 gene. Similar phenotypes could be observed between overexpression of the candidate miRNAs and siRNA targeting the Nrf2 gene with respect to the expression of the Nrf2 gene both at mRNA and protein level, ROS accumulation, cellular proliferation, mitochondrial activity and the expression of antioxidants. Over accumulation of intracellular ROS level was associated with reduction in mitochondrial activity (Saeed-Zidane et al. 2017). Our results were in agreement with the aforementioned results, where overexpression of miR-153, miR-28 and miR-708 led to an increment in the level of intracellular ROS and impaired mitochondrial activity accompanied by a reduction in cellular proliferation. Overexpression of candidate miRNAs under oxidative stress condition showed a reduction in Nrf2 mRNA and protein levels resulted in increasing intracellular ROS level and reduced mitochondrial activity which led to lower cellular proliferation compared to H_2O_2 alone treated group. This has evidenced the potential of

modulating the Nrf2 mediated oxidative stress response mechanisms in bovine granulosa cells by inducing the expression of the candidate miRNAs. The potential of this approach in therapeutic applications could be the focus of future research.

Oxidative stress affects cattle reproduction and several metabolic processes in transition cows. Supplementation of antioxidants is reported to induce protective effects against oxidative stress conditions and restores cellular antioxidant defense mechanisms (Poljšak and Fink 2014), which showed beneficial effect on the quality of bovine meat and milk production (Castillo et al. 2013). Quercetin is a member of polyphenolic compounds known as flavonoids. Accumulating evidence demonstrated that Que have an antioxidant activity against oxidative stress in different cell types (Kumar Mishra et al. 2013; Choi et al. 2015). Furthermore, dietary supplementation of Que in heat stressed rabbit showed improved follicular development, reduced apoptosis in granulosa cells and maintained oocyte competence (Naseer et al. 2017). Several studies showed that Que exert an anti-inflammatory effect through inhibition of NF-κB and activation of Nrf2 (Kang et al. 2013; Saw et al. 2014; Sun et al. 2015). In the present study, supplementation of Que to cultured granulosa cells resulted in increased transcription of Nrf2 in dose dependent manner. Low dose of Que has resulted in increasing Nrf2 at mRNA and protein levels accompanied by increased cellular proliferation and mitochondrial activity. However, higher concentrations of Que resulted in increased intracellular ROS level, reduce the mitochondrial activity and induced S-phase cell cycle arrest. These findings are in agreement with previous reports on the impact of Que on Nrf2 in different cellular functions in various cell models (Arredondo et al. 2010; Pallauf et al. 2017; Kim et al. 2018). On the other hand, Que has the capability to act as pro or antioxidant depending on concentration and cellular model (Robledinos-Antón et al. 2017). The antioxidant activity of Que was reported at low concentration, while higher concentrations resulted in decrease cellular proliferation as a result of induced apoptosis (Robaszkiewicz et al. 2007), stimulate the generation of superoxide radicals ($O_2^{\bullet-}$) and subsequently affecting mitochondrial activity (Marchi et al. 2009). Moreover, Que is reported inducing a cell cycle arrest at G0/G1, S-phase or G2/M depending on cell types (Yuan et al. 2012; Richter et al. 1999; Zhang et al. 2008; Suh et al. 2010). Similarly, our findings showed that Que treatment at lower concentration resulted in cellular viability enhancement, reduction of intracellular ROS level and increased mitochondrial activity, as it has been reported before (Zhao et al. 2014; Wang et al. 2014; Bao et al. 2017). Que is

known to modulate expression of Nrf2 through multiple pathways including transcriptional regulation, posttranscriptional through stabilizing Nrf2 protein and inhibiting Nrf2 ubiquitination and posttranslational level through Keap1 modification (Tanigawa et al. 2007). Similarly, our results revealed for the first time that Que could modulate the expression of miR-153, miR-28/708 expression pattern. This could show the indirect effect of Que on Nrf2 activity in addition to its direct effect. Such mechanisms of action of flavonoids including Que has been reported in vivo and in vitro experimental setups (Boesch-Saadatmandi et al. 2011; Boesch-Saadatmandi et al. 2012; Zhang et al. 2015; Del Follo-Martinez et al. 2013; Chuammitri et al. 2017). Taken all results together a hypothetical mechanism of both endogenous and exogenous modulation of the Nrf2 mediated oxidative stress defense mechanism in bovine granulosa cells is illustrated in figure 2.10.
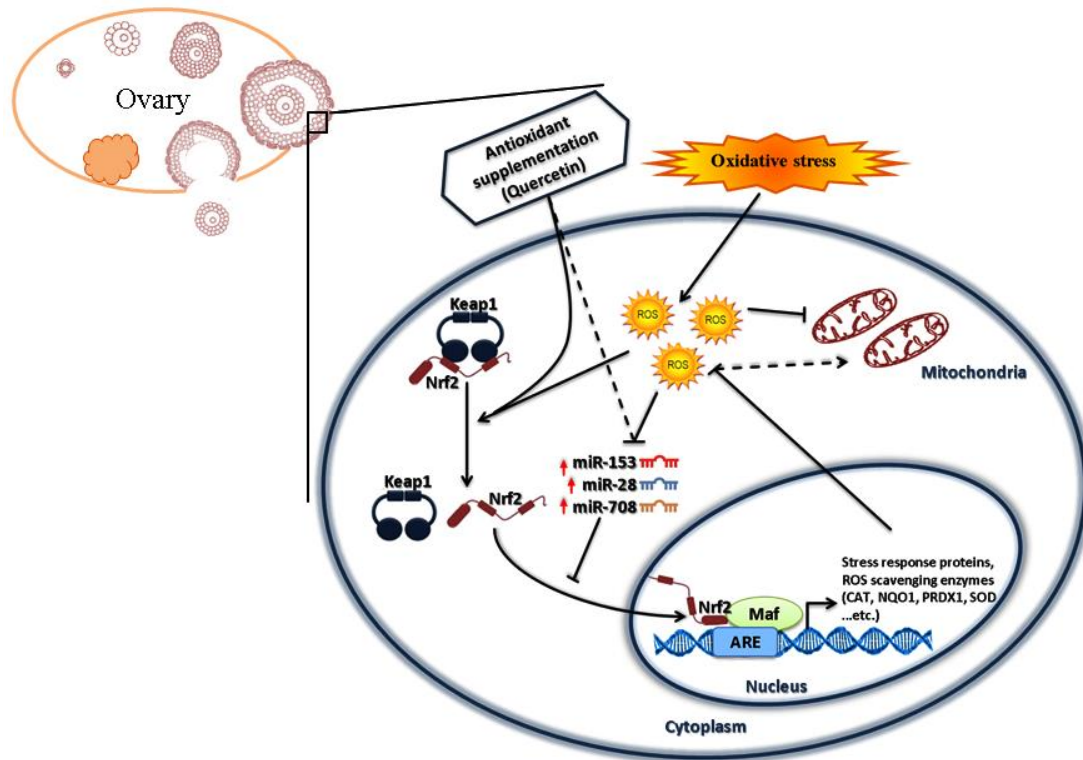
Figure 2.10. Model of the Nrf2-mediated oxidative stress response pathway in bovine granulosa cells under moderate oxidative stress condition and under antioxidant (quercetin) supplementation. Oxidative stress and Que modulate Nrf2 posttranscriptionally through the inhibition of miR-153, miR-28 and miR-708 which targeted bovine Nrf2. After that, Nrf2 protein was activated and localized to the nucleus, where it binds to the antioxidants reactive element (ARE) in the promoters of antioxidant genes. This activates antioxidant gene expression so the respective proteins can scavenge the excessive ROS and subsequently maintain the activity of mitochondria.

2.5. Materials and Methods

2.5.1. Bovine granulosa cell culture

Bovine ovaries were collected and transported from local slaughterhouse in thermo-flask containing warm (37 ^{O}C) physiological saline (NaCl 0.9 %) solution. Upon arrival, ovarian sample were washed with calcium and magnesium free phosphate buffer saline (PBS-CMF) three times followed by rinsing in 70 % ethanol for 30 seconds. Thereafter, the follicular fluid was aspirated from small growing follicles (3-5 mm diameter) using a 20-gauge needle and collected on pre-warmed PBS-CMF in 15 ml tube. After collection, the aspirated follicular fluid was kept in standing position for 15 min at 37 ^{O}C to allow the

cumulus-oocyte-complex (COC) to settle at the bottom of the tube. The upper supernatant part containing the granulosa cells were transferred to another 15 ml tube and centrifuged at 750 rpm for 7 minutes. The pellets were resuspended in red blood cells (RBCs) lysis buffer for 1 minute, followed by addition of DMEM/F12-HAM (Sigma-Aldrich, München, Germany) to stop lysis buffer reaction and centrifuged at 500 rpm for 7 min. Trypan blue exclusion method was used to determine cell viability and concentration. Cells were cultured at rate of 2.5 x 10^5/well in CytoOne® -24 well plate (Starlab International GmbH, Hamburg, Germany) in 500 µl DMEM/F12-HAM supplemented with 10 % fetal bovine serum (FBS) (Gibco FBS, Life technologies, Schwerte, Germany), 100 IU/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml fungizone (Sigma-Aldrich, München, Germany) and incubated at 37 ^{O}C in and 5 % CO_2.

2.5.2. MicroRNA target gene prediction and luciferase reporter assay

Prediction of miRNAs targeting Nrf2 was done using TargetScan; an online target prediction database (http://www.targetscan.org), miRNAs (miR-153, miR-28 and miR-708) were selected based on the probability of preferential conservation (Friedman et al. 2009; Agarwal et al. 2015).

The interactions between miR-153, miR-28 and miR-708 and Nrf2 gene were validated using luciferase reporter assay as described in Gebremedhn et al (Gebremedhn et al. 2015). Briefly, DNA fragments containing the putative miRNA binding sites in the 3´-UTR of Nrf2 gene (wild type) for the aforementioned miRNAs were amplified from bovine genomic DNA. Similarly, DNA fragment containing mutations at the binding sites (mutant) were separately designed. The designed wild type and mutant fragments were cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega GmbH, Mannheim, Germany) between the *SacI* and *XhoI* restriction sites (Figure 2.S5). The presence of the miRNA binding sites in the wildtype construct and the absence of the binding sites in the mutant constructs were confirmed by sequencing the PCR amplicon of the pmirGLO vector.

Following this, sub-confluent in vitro cultured granulosa cells (70-80 % of confluency) were co-transfected with 350 ng of plasmid containing either wild-type or mutant sequence with 100 nM of the corresponding miRNA mimics (Exiqon, Vedbaek, Denmark) using Lipofectamine® 2000 transfection reagent (Life Technologies, Germany). Twenty-four

hours later, cells were lysed using 1x Passive Lysis Buffer (Promega GmbH, Germany) and the firefly and Renilla Luciferase activities were determined based on the Dual-Luciferase® Reporter (DLR™) Assay System (Promega GmbH, Germany) according to manufacturer's protocol. The absorbance of firefly and Renilla luciferase activity were measured using Centro LB 960 Microplate Luminometer (Berthold Technologies GmbH, Bad Wildbad, Germany). Data was analyzed as the ratio of firefly to Renilla activity.

2.5.3. MicroRNA and siRNA transfection

To determine the impact of candidate miRNAs in modulating the expression of Nrf2, candidate miRNAs were either overexpressed or inhibited using chemically synthetized miRNA mimics and inhibitors (Exiqon, Denmark), respectively. For this, 100 nM miRNA mimic, inhibitor, or the corresponding negative control (NC) were transfected in sub-confluent granulosa cells using Lipofectamin® 2000 (Invitrogen, Carlsbad, CA) transfection reagent in Opti-MEM I reduced-serum medium (Invitrogen, Carlsbad, CA). Twenty-four hours post-transfection, cells were subjected to mRNA and protein expression analysis. Moreover, detection of intracellular ROS level, cell proliferation assay and assessment of mitochondrial activity were performed.

In order to cross-validate the regulatory role of candidate miRNAs on Nrf2 gene functions, targeted knockdown of Nrf2 was performed using bovine specific siRNA (Exiqon, Denmark). For this, sub-confluent granulosa cells were transfected with 200 nM of siRNA -Nrf2 or siRNA negative control (NC) using Lipofectamin 2000 in Opti-MEM I reduced-serum medium. Twenty-four hours post-transfection, the cells were subjected to intracellular ROS level detection, cell proliferation assay, assessment of mitochondrial activity, mRNA and protein expression pattern analyses.

2.5.4. Exogenous induction of Nrf2 by quercetin

Stock solutions of Que were freshly prepared by dissolving it in Dimethyl sulfoxide (DMSO). The stock solutions were subsequently diluted with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12-HAM) media. The final concentrations of DMSO in the medium were $\leq 0.01\%$ (v/v). Appropriate controls with only the vehicle DMSO were included in all experiments. For exogenous Nrf2 modulation, the appropriate and non-toxic dose of Que was determined. For that, sub-confluent granulosa cells were

treated with different concentrations of Que (0, 10, 20, 50, 100 or 200 µM) for 24 hours. Treated cells were then subjected to intracellular ROS level detection, cell cycle assay, cell proliferation assay and assessment of mitochondrial activity. Moreover, mRNA and protein expression patterns of candidate genes were quantified in each treatment group.

2.5.5. Induction of oxidative stress using H_2O_2

The effects of miR-153, miR-28 and miR-708 overexpression and the rescuing role of Que on bovine granulosa cells when subjected to oxidative stress condition were investigated. For this, cells were treated with 5 µM H_2O_2 for 40 min (Saeed-Zidane et al. 2017), then transfected with miRNA mimics or supplemented with 10µM Que. Twenty-four hour post-treatment, cells were subjected to intracellular ROS level, mitochondrial activity, cellular proliferation rate and Nrf2 expression pattern analyses.

2.5.6. Total RNA isolation and quantitative real-time PCR (qRT-PCR)

Cells were harvested 24 hours post treatment (miRNA, siRNA transfection and que supplementation), subjected to total RNA isolation using miRNeasy® mini kit (Qiagen GmbH, Hilden, Germany) following manufacturer's protocol. After assessing the quality and concentration of the RNA samples using NanoDrop 8000 spectrophotometer (NanoDrop technologies, Schwerte, Germany), cDNA synthesis was performed using first stand cDNA synthesis kit (Thermo Fisher scientific, Schwerte, Germany). Briefly, RNA concentration was adjusted using nuclease-free water to total volume 10 µl from each replicate was followed by co-incubation with 0.5 µl oligo (dT)18 and 0.5 µl random primer at 65 ^{O}C for 5 min. Next, 1 µl RiboLock, 4 µl 5x reaction buffer, 2 µl dNTPs and 2 µl reverse transcriptase were added for each sample and co-incubated at 25 ^{O}C for 5 min, 37 ^{O}C for 60 min, and 70 ^{O}C for 5 min. After incubation, samples were stored at -20 ^{O}C till gene expression analysis.

The relative abundance of Nrf2 and its downstream antioxidant genes; NQO1, PRDX1, SOD1 and CAT was quantified using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany) in Applied Biosystem® StepOnePlus™ (Applied Biosystems, CA, USA) using gene specific primers (Table 2.S1). All primers were designed using NCBI primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Data was analyzed using the comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and

Schmittgen 2001) and the average expression levels of ACTB and GAPDH was used for normalization.

For miRNA expression analysis, cDNA was synthetized using miRCURY® LNA® RT kit (Qiagen GmbH, Germany) following the manufacturer's protocol. Briefly, 80 ng of miRNA-enriched total RNA was used for cDNA synthesis. Reverse transcription master mix of 2 µl reaction buffer, 1 µl enzyme mix, and complete reaction volume up to 10 µl. Thereafter, incubate samples at 42 $^{\mathrm{O}}$C for 60 min and 95 $^{\mathrm{O}}$C for 5 min to inactivate the reverse transcription enzyme. Synthetized cDNA was 15x diluted and used for RT-PCR analysis using miRCURY LNA SYBR® Green PCR kit (Qiagen GmbH, Germany) following the manufacturer's protocol. The thermal cycler was programmed for initial preheating at 95 $^{\mathrm{O}}$C for 2 min, followed by 40 cycles of amplification at 95 $^{\mathrm{O}}$C for 10 sec and 56 $^{\mathrm{O}}$C for 60 sec followed by melting curve analysis. Data was analyzed using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schmittgen 2001) and the expression level of 5S ribosomal RNA and U6 were used as internal control for normalization.

2.5.7. Cell proliferation assay

To determine the impact of modulation of Nrf2 by the candidate miRNAs on cell proliferation, 1.5×10^4 granulosa cells were seeded into 96-well plate and cultured in the F-12 media. Sub-confluent granulosa cells were then transfected with miRNA, siRNA or treated with different concentration of Que. Twenty-four hours post-treatment, 10 µl of CCK-8 kit solution (Dojindo EU GmbH, München, Germany) was added into each well and the plate was incubated for 4 h at 37 $^{\mathrm{O}}$C in and 5 % CO_2. The optical density (OD) was at a wavelength 450 nm using Synergy™ H1 Multi-Mode Reader (BioTek, Bad Friedrichshall, Germany). OD from empty wells was used for background correction.

2.5.8. Protein immunofluorescence detection

The abundance and localization of the Nrf2 protein in granulosa cells subjected to transfection with miRNA, siRNA and Que was determined using immunocytochemistry. Briefly, cells cultured in 8-well chamber slide. 24 h post treatment, cells were washed with PBS-CMF, and then fixed overnight at 4 $^{\mathrm{O}}$C in 4 % (w/v) paraformaldehyde in PBS-CMF. Fixed cells were washed three times with PBS-CMF, then permeabilized with 0.3 % (v/v) Triton-X100 (Sigma-Aldrich) for 10 min at room temperature followed by washing with

PBS-CMF 3 times for 5 minutes. Cells were incubated in 4 % donkey serum (Sigma-Aldrich) for 1 h at room temperature, followed by incubation overnight at 4 ^{O}C with polyclonal rabbit primary antibodies against Nrf2 (1:100, orb11165, Biorbyt, Cambridge, UK). Then, cells were further incubated at 37 ^{O}C for 3 hr in the dark with fluorescence-labelled secondary antibody (Alexa flour™ 568 goat anti-rabbit 1:350, Life Technologies, Germany). A droplet of Vectashield mounting medium containing DAPI (Dabco; Acros, Geel, Belgium) was used to stain the nuclei. Finally, images were visualized under a CLSM LSM-780 confocal lasers scanning microscope (Carl Zeiss GmbH; Jena, Germany) and analyzed using ImageJ 1.48v (National institutes of Health, Maryland, USA, https://imagej.nih.gov/).

2.5.9. Assessment of mitochondrial activity

The mitochondrial activity in bovine granulosa cells transfected with miRNA, siRNA and Que was assessed using MitoTracker® Red CMXRos (M7512; Invitrogen) according to manufacturer´s instructions. Breifly, lyophilized MitoTracker® product was dissolved in a high-quality, anhydrous dimethylsulfoxide (DMSO) to a final concentration of 1 mM. The final working concentration (200 nM) was prepared by diluting stock solution in DMEM/F12-HAM medium. MitoTracker® Red CMXRos is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential (MMP), which is a marker for mitochondrial functionality (Sakamuru et al. 2016; Poot et al. 2017). For that, granulosa cells were cultured in 8-well slide and cells were incubated with 200 nM MitoTracker® red dye at 37 ^{O}C for 30 min, followed by two washing with PBS-CMF and then fixed overnight at 4 ^{O}C with 4% paraformaldehyde. Fixed cells were mounted with Vectashield (H-1200) containing DAPI. Images were acquired at 40x magnification under a CLSM LSM-780 confocal laser-scanning microscope (Carl Zeiss GmbH; Germany) and analyzed using ImageJ 1.48v (National institutes of Health, Maryland, USA, https://imagej.nih.gov/).

2.5.10. Cell cycle assay

The cell cycle status of cells treated with different doses of Que was determined using propidium iodide (PI) staining in flow cytometer as previously described in (Gebremedhn et al. 2016; Andreas et al. 2016; Pande et al. 2018). Briefly, cells were trypsinized 24 hours after treatment. Following cell counting, a minimum of $1x10^6$ cells were fixed overnight at

4 ^{O}C in ice-cold 70 % ethanol. Fixed cells were stained with 50 µg/ml PI (Invitrogen, Carlsbad, CA) and 50 μg/ml RNase and readings were acquired in flow cytometer BD Biosciences FACS Calibur, CA, USA). A minimum of 10,000 cells were acquired per sample and data were analyzed using ModFit LT software (http://www.vsh.com/products/mflt/index.asp).

2.5.11. Intracellular ROS detection

Cultured cells in each experiment were subjected to ROS accumulation assay using 2´,7´-dichlorofluorescin diacetate (H2DCFDA) (Life Technologies, Germany) according to manufacturer´s instructions. Cells were incubated with 50 µl of 75 µM H2DCFDA diluted in PBS-CMF for 20 min in dark at 37 ^{O}C. Following this, cells were washed twice in PBS-CMF, and images were captured immediately under an 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, Maryland, USA, https://imagej.nih.gov/).

2.5.12. Statistical analysis:

Data were analyzed using Graphpad prism 5 (Graphpad, San Diego, CA) and presented as mean ± SEM of at least three biological replicates. Statistical significance between mean values of more than two treatment groups was determined using one-way analysis of variance (ANOVA) followed by Tukey multiple pairwise comparison. Moreover, data from two treatment groups were analyzed using student´s two-tailed t-test. The statistical significance was determined at p=0.05.

2.6. Conclusions

In conclusion, the present study evidenced the mechanisms of regulation of Nrf2 mediated oxidative stress response pathway in bovine granulosa cells and indicate the potential application of those regulatory mechanisms in future fertility treatment strategies to enhance ovarian functionality.

2.7 Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/
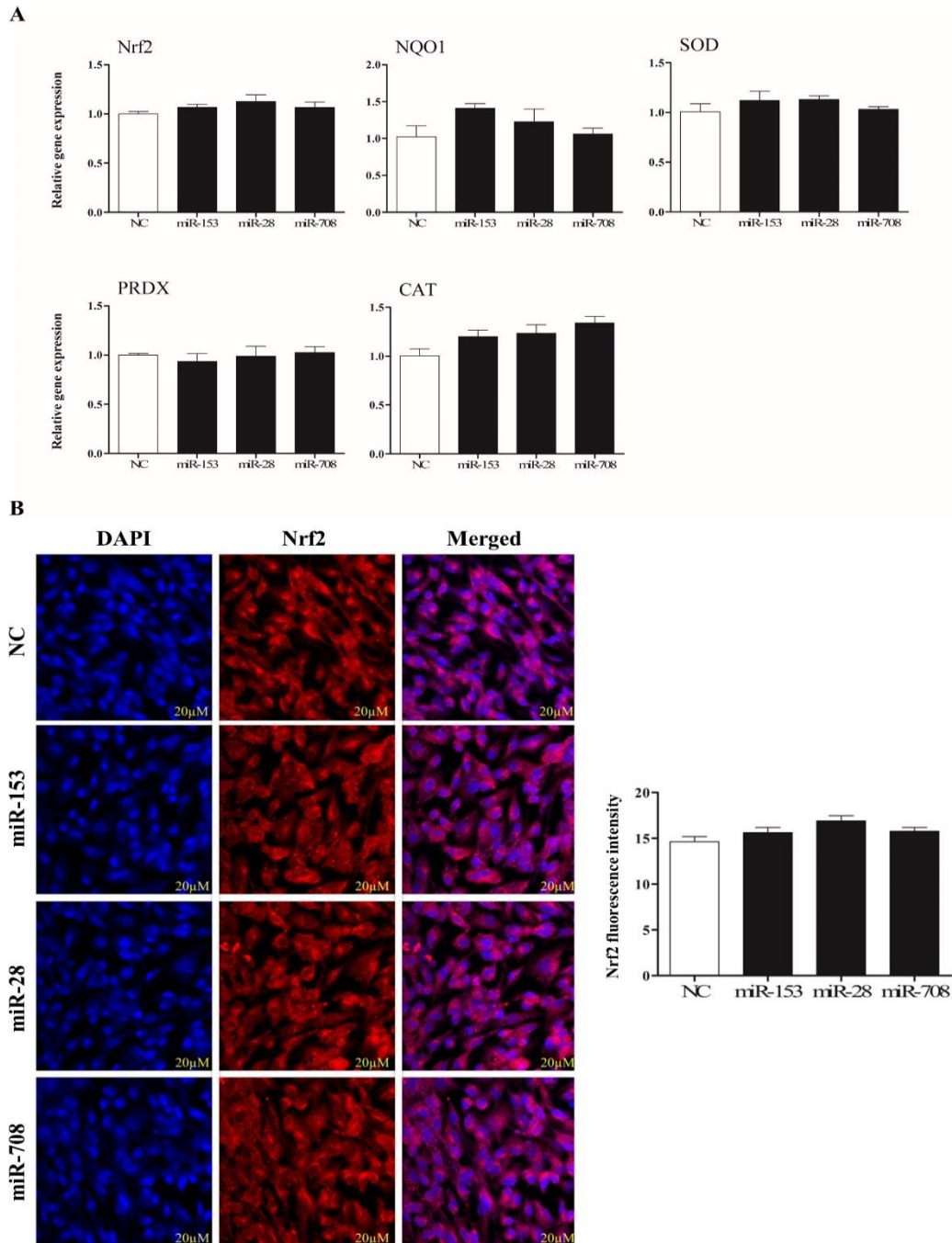
Figure 2.S1. Quantitative RT-PCR of Nrf2 and its downstream antioxidant targets in bovine granulosa cells transfected with miR-153, miR-28 and miR-708 inhibitors (**A**). Immunocytochemistry of Nrf2 in bovine granulosa cells transfected with candidate miRNAs inhibitors (**B**). White bars represent granulosa cells transfected with inhibitor negative control (NC) and dark bars represent cells transfected with miRNA inhibitors. Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b) showed statistically significant differences ($p < 0.05$).
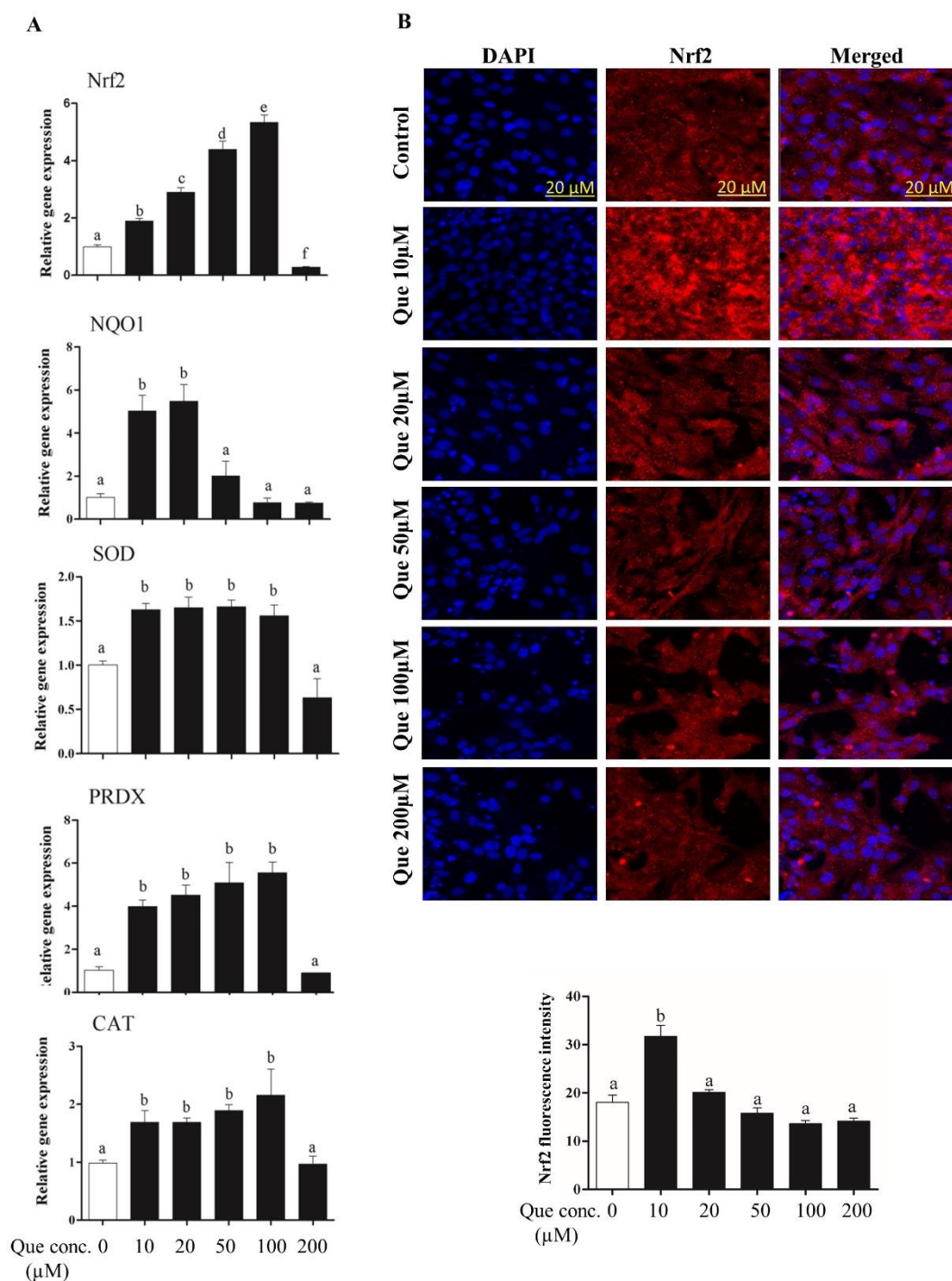
Figure 2.S2. Quercetin modulates the mRNA expression level of Nrf2 and its downstream antioxidants. Quantitative RT-PCR of Nrf2 and its down stream antioxidants in bovine granulosa cells control group (white bar) or different Quercetin concentrations (black bar) (**A**). Immunocytochemistry of Nrf2 in bovine granulosa cells co-incubated with different Que concentration (0 – 200 µM) (**B**). Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b,c,d,e,f) showed statistically significant differences (p<0.05).
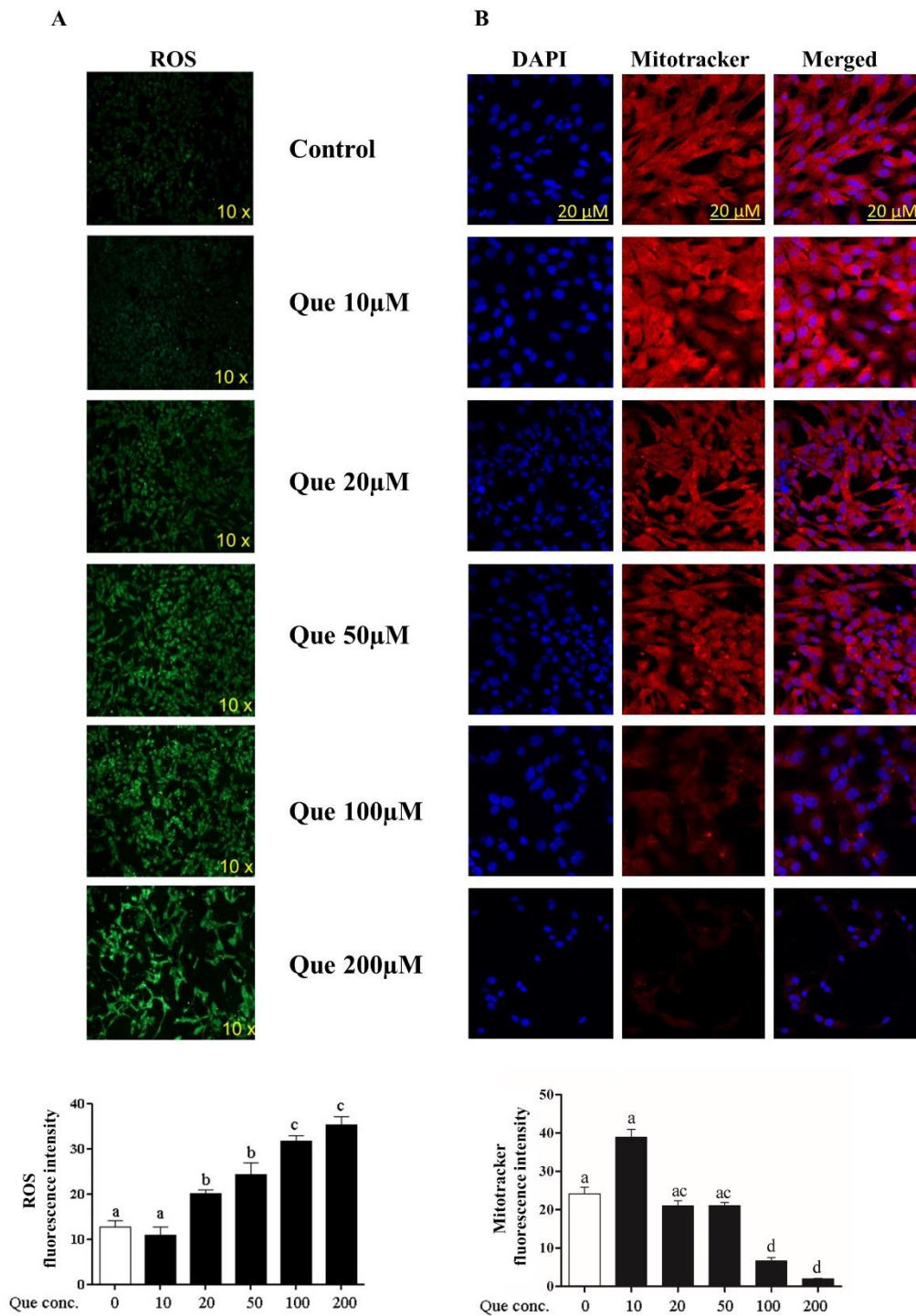
Figure 2.S3. Quercetin increases intracellular ROS accumulation levels in dose dependent manner (**A**). Supplementation of quercetin at 10μM increased mitochondrial activity. However, higher concentration of quercetin reduced the mitochondrial activity (**B**). Data are presented as mean ±SEM of three independent biological replicates. Bars with different letters (a,b,c,d) showed statistically significant differences (p<0.05)
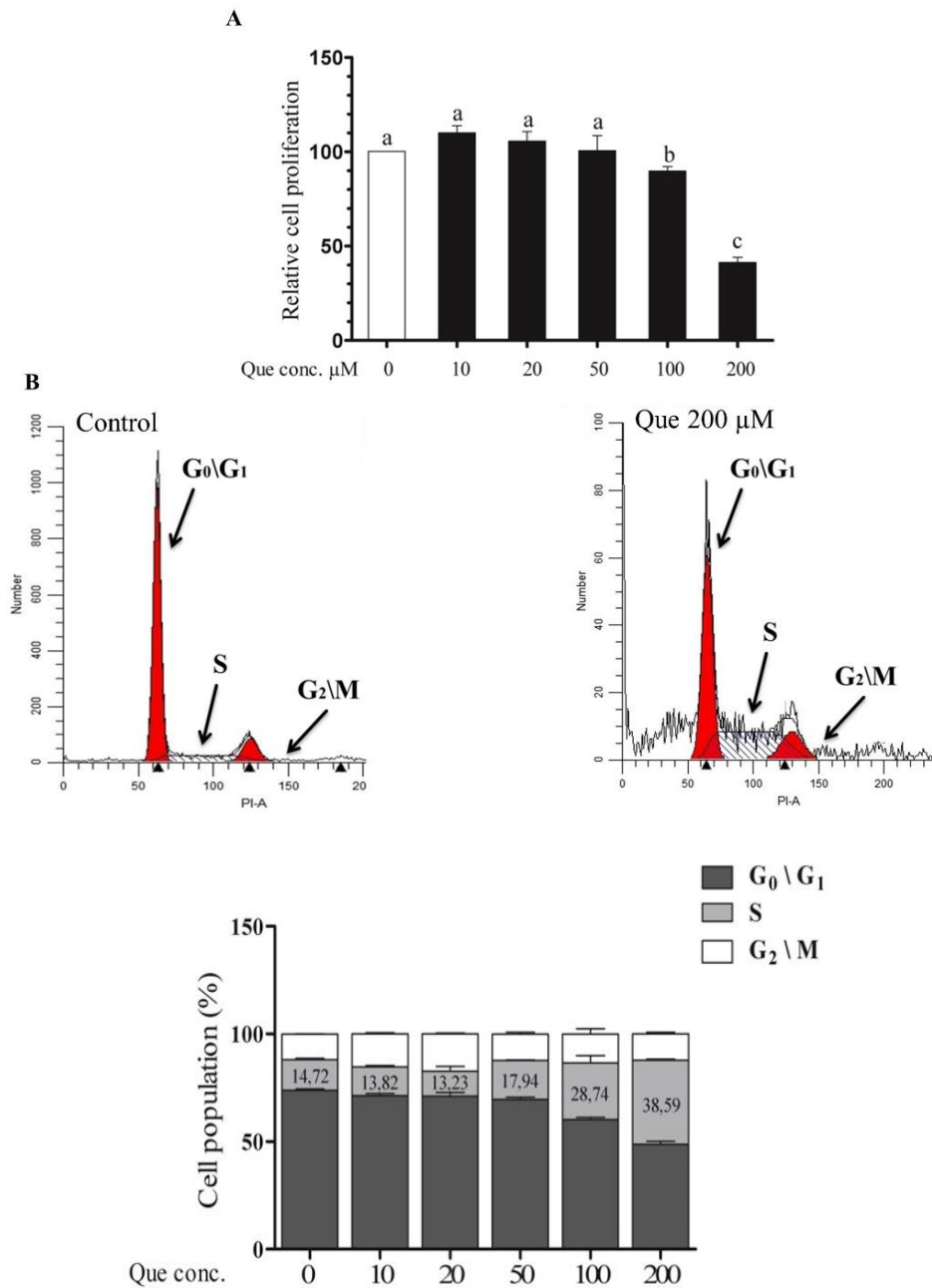
Figure 2.S4. Supplementation of higher concentrations of quercetin resulted in reduced bovine proliferation of granulosa cells (**A**). Flow cytometric analysis showed a shift in cell cycle transition towards the S-phase (**B**). Data are presented as mean $\pm$SEM of three independent biological replicates. Bars with different letters (a,b) showed statistically significant differences ($p<0.05$)
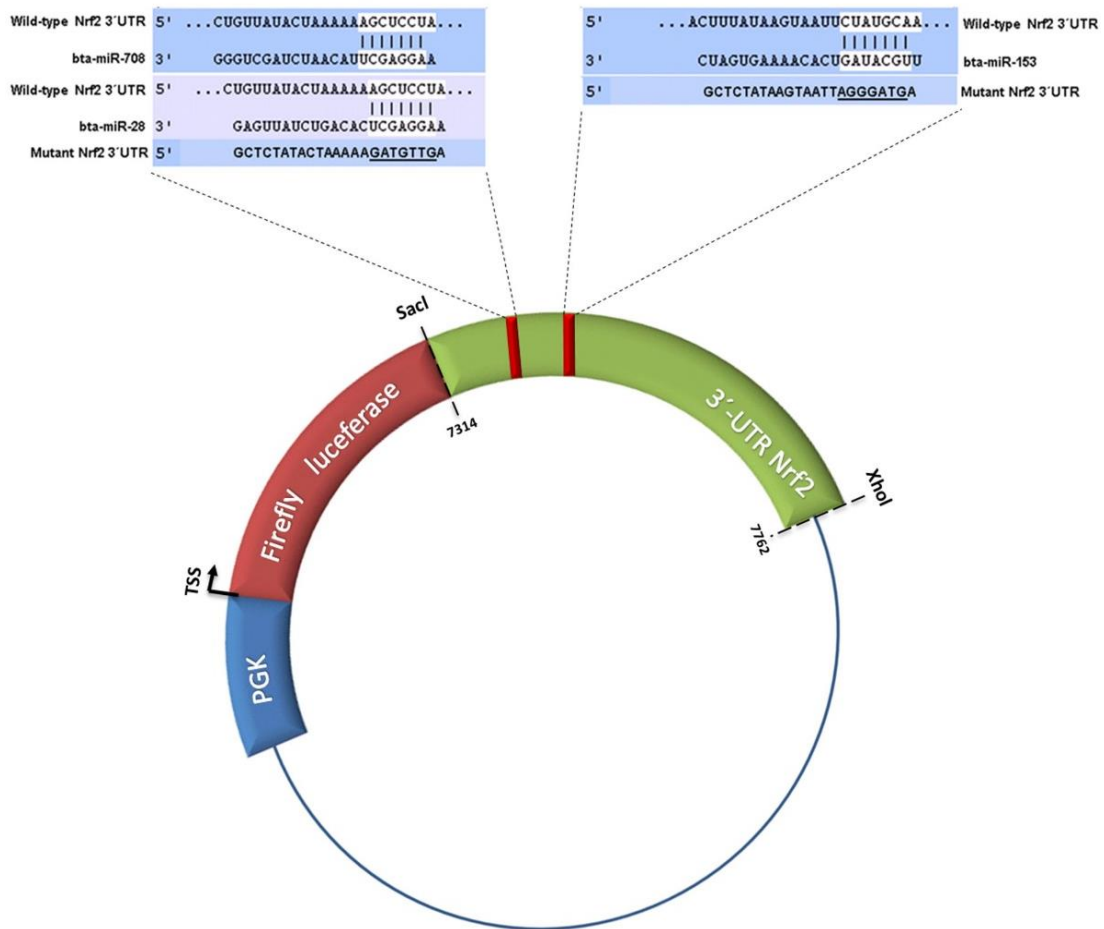
Figure 2.S5. MiRNAs coordinately target Nrf2 mRNA in bovine granulosa cells. Putative binding sites of miR-153, miR-28 and miR-708 and their genomic coordinates in the 3´-UTR of bovine Nrf2 mRNA are indicated. Plasmid with wild-type and mutant sequences (underlined) for the miRNA binding were fused into the downstream multiple cloning sites of the firefly luciferase gene between the SacI and XhoI restriction sites. The PGK upstream promoter and transcription start site (TSS) are indicated in blue and arrow, respectively.

Table 2.S1. The list of primers and their sequences of selected candidate genes used for qRT-PCR analysis

Gene	Primer sequence	Size (bp)	Accession number
GAPDH	F: 5´-ACCCAGAAGACTGTGGATGG-3´ R: 5´-ACGCCTGCTTCACCACCTT-3´	247	NM_001034034
B-ACTIN	F: 5´-GGCATTCACGAAACTACCTT-3´ R: 5´-CAATCCACACGGAGTACTTG-3´	208	NM_173979
Nrf2	F: 5´-CCCAGTCTTCACTGCTCCTC-3´ R: 5´-TCAGCCAGCTTGTCATTTTG-3´	165	NM_001011678
NQO1	F: 5´-AACCAACAGACCAGCCAATC-3´ R: 5´-CACAGTGACCTCCCATCCTT-3´	154	NM_001034535.1
SOD1	F: 5´-TGCCATCGTGGATATTGTAG-3´ R: 5´-GCAATTCCAATTACACCACA-3´	174	NM_174615
PRDX1	F: 5´-TGGATCAACACACCCAAGAA-3´ R: 5´-GTCTCAGCGTCTCATCCACA-3´	217	NM_174431.1
CAT	F: 5´-TGGGACCCAACTATCTCCAG-3´ R: 5´-AAGTGGGTCCTGTGTTCCAG-3´	178	NM_001035386.1

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3

Quercetin modulates Nrf2-mediated oxidative stress response in bovine preimplantation embryos

Omar Khadrawy[1], Samuel Gebremedhn[1], Dessie Salilew-Wondim[1], Mohamed Omar Taqi[1], Franca Rings[2], Christiane Neuhoff [1], Ernst Tholen[1], Eva Held[1,2], Michael Hoelker[1,2,3], Karl Schellander[1,3], Dawit Tesfaye[1,3]

[1]Institute of Animal Science, Department of Animal Breeding and Husbandry, University of Bonn, Bonn, Germany

[2]Teaching and Research Station Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany

[3]Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

*Correspondence to:

Dr. Dawit Tesfaye, PhD

Department of Ainmal Breeding and Husbandry

Institute of Animal Science

Endenicher Allee 15,

Bonn 53115, Germany

E-mail: tesfaye@itw.uni-bonn.de

3.1 Abstract

In vitro-derived embryos exposed to oxidative stress induced by high oxygen tension. Nrf2 is a master regulator for antioxidant machinery against oxidative stress in bovine preimplantation embryos, which was found to be regulated via epigenetic mechanisms including miRNAs. In the present study we investigate the protective effect of quercetin on preimplantation embryos quality and development with respect to Nrf2 signaling pathway. For that, blastocysts, which developed from zygotes cultured in media supplemented with or without quercetin under high oxygen level (20 %), were subjected to determination of intracellular ROS level, mitochondrial activity, blastocyst formation rate and total cell number. The mRNA expression level of Nrf2 and its downstream antioxidant genes, Nrf2 protein abundance and the pattern of miRNAs targeting Nrf2 were also investigated. Quercetin supplementation in culture media shows no effect on cleavage as well as developmental rate. Quercetin supplementation resulted in increasing the blastocyst total cell number and reduction of intracellular ROS level accompanied with increasing mitochondrial activity compared to control group in both day 7 and day 8 blastocysts. Moreover, quercetin supplementation induced Nrf2 at transcriptional and\or translational level and resulted in activation of cellular antioxidant machinery. In conclusion, quercetin protects preimplantation embryos against oxidative stress and improves embryo quality through activating Nrf2 signaling pathway.

3.2 Introduction

During the course of in vitro embryo production, preimplantation embryos are constantly exposed to multitudes of environmental stressors originating from the culture conditions. Embryos obtained from the in vitro system are different from the in vivo counterparts in many aspects (Lonergan et al. 2003). The main difference between the two culture conditions is attributed to higher oxygen tension of the in vitro culture (20 % O_2), which is much higher than the 2-8 % O_2 in oviduct and uterus of most mammalian species (Catt and Henman 2000; Fischer and Bavister 1993). Exposure to highe oxygen concentration within the in vitro culture environment induces oxidative stress, which results in the production of excessive reactive oxygen species (ROS) (Fischer and Bavister 1993; Amin et al. 2014; Leite et al. 2017). A considerable number of evidences showed that, high oxygen tension and other suboptimal culture conditions affect the expression of transcription factors involved in pathways related to oocyte maturation and quality (Chaube et al. 2014), embryo development, quality and competency (Gad et al. 2012; Amin et al. 2014; Leite et al. 2017). Under in vitro embryo production system, various intrinsic and extrinsic factors are responsible for the excessive ROS production (Agarwal et al. 2014). The embryo itself is considered as an endogenous source of ROS (Guérin et al. 2001). The aerobic metabolisms of oxidative phosphorylation and glycolysis are utilized to meet energy demand of the embryo, which in turn generates excessive ROS and cause oxidative stress (Thompson et al. 2000). On the other hand, exogenous factors like visible light (Beehler et al. 1992; Takenaka et al. 2007), culture media (Guérin et al. 2001), pH (Will et al. 2011) and temperature (Suzuki and Mittler 2006) could lead to increased ROS production. Maintaining the intracellular ROS at homeostatic level is vital for normal embryo development (Agarwal et al. 2014). Growing evidences documented the deleterious effect of excessive ROS on embryo development (Shih et al. 2014; Amin et al. 2014; Chen et al. 2017). Oxidative stress damages cellular components including DNA, lipid, proteins, mitochondria and endoplasmic reticulum leading to DNA fragmentation, alteration of DNA methylation pattern, changes in the expression of genes and metabolic activities (Burroughs et al. 2013; Amin et al. 2014; Yoon et al. 2014; Li et al. 2016a).

The Nrf2 signaling pathway is considered to be the master regulator of antioxidants defense mechanism (Kensler et al. 2007), which regulates a battery of 250 genes involved in variety of cellular function including cytoprotection against endogenous and

environmental stressors (Chanas et al. 2002; Kwak et al. 2003; McMahon et al. 2001; Hayes and Dinkova-Kostova 2014). Under physiological conditions, Nrf2 is kept in the cytoplasm and maintained at lower levels by an inhibitory protein; Keap1 (McMahon et al. 2003). However, oxidative stress conditions dissociates the Nrf2 from Keap1 and subsequently translocates into the nucleus and binding to a specific DNA sequence known as antioxidant response element (ARE); in upstream promoter of its downstream antioxidant target genes superoxide dismutase (SOD1), NAD(P)H:quinone oxidoreductase (NQO-1) and catalase (CAT) (Itoh et al. 1997b; Kensler et al. 2007; Kalayarasan et al. 2009). Moreover, antioxidant supplementation to in vitro culture media resulted in reduction of intracellular ROS accumulation in oocytes and preimplantation embryos (Yu et al. 2014; Sovernigo et al. 2017).

Supplementation of antioxidant into the in vitro culture media is reported to endow the preimplantation embryos with protective potential against oxidative stress damage. Quercetin (Que), a plant-derived flavonoids mainly polyphenolic compound found in fruits and vegetables, has been reported to have an antioxidative (Naderi et al. 2003; Yang et al. 2014; Kim et al. 2006; Bao et al. 2017), anti-inflammatory (Kang et al. 2013a; Saw et al. 2014; Sun et al. 2015) and anti-apoptotic effect (Yang et al. 2014). Que is considered as candidate chemopreventive against oxidative stress in different cell types (Chun et al. 2014), by modulating several signaling pathways mainly the Nrf2 (Murakami et al. 2008; Ji et al. 2015) through various mechanisms by stabilizing Nrf2 protein activity and inhibiting Nrf2 ubiquitination (Tanigawa et al. 2007). Previous studies indicated that the polyphenolic compound interact with cellular antioxidants defense system such as NQO1, glutathione S-transferase, thioredoxin and heme oxygenase 1 (Ghanim et al. 2011). Here, we aimed to investigate the protective role of quercetin in bovine preimplantation embryos against oxidative stress induced by high atmospheric oxygen level and to explore the underlying activation of cellular antioxidant mechanisms.

3.3. Materials and methods

3.3.1. Experimental design

The aim of our study was to elucidate the protective effect of quercetin to improve the development and quality of bovine preimplantation embryos cultured at high atmospheric oxygen level (20%) with respect to Nrf2 signaling pathway. For that, bovine oocyte cumulus complex was aspirated from small to medium sized follicles (3-5 mm diameter) and cultured in modified TCM-199. Matured oocytes were fertilized; presumptive zygotes were cultured in SOFaa culture medium supplemented with or without 10 µM quercetin under 20 % O_2 level. Cleavage and blastocyst formation rate were investigated. Moreover, day 7 and day 8 blastocysts were subjected to genotype and phenotypes analysis to figure out the protective effect of quercetin on bovine preimplantation embryos through Nrf2 signaling pathway.

3.3.2. Oocyte collection and in vitro maturation

Bovine ovaries were collected from local slaughterhouse and transported to the laboratory in thermo-flask containing warm physiological saline (0.9% NaCl). Ovaries were washed by phosphate buffered saline, calcium and magnesium free (PBS-CMF), rinsed in 70% ethanol two times for 1 min and washed again by PBS-CMF. Follicular fluid from medium-sized follicles (3-8 mm diameter) was aspirated using a 20-gauge needle and collected in pre-warmed PBS-CMF. Only oocyte surrounded by a multilayer of compacted cumulus cells and showing homogenous cytoplasm were transferred to in vitro maturation (IVM) medium (M-2154; Sigma-Aldrich, Munich, Germany) supplemented with 50 mg/ml gentamicin, 2 mM pyruvate, 33.9 mM NaCHO3, 2.9 mM calcium lactate, 4.4 mM HEPES, 2 % heat-inactivated oestrus cow serum (OCS) and 10 µg/mL FSH. The COCs were cultured at 39 °C and 5 % CO2. Maturation of oocytes were assessed based on the expansion of cumulus cells and presence of the first polar body.

3.3.3. In vitro fertilization, embryo production and quercetin supplementation

A group of 50 matured COCs were co-incubated with of 2×10^6 sperm/ml bull sperm in Fert-TALP medium supplemented with 10 mM hypotaurine, 2 mM noradrenaline, 20 mM penicillinamine, 6 mg/ml bovine serum albumin (BSA), 50 mg/ml gentamicin, and 1

mg/ml heparin for 18 hours at 39 °C and 5 % CO_2 in air. Presumptive zygotes were cultured in 400 µl of SOFaa culture medium supplemented with or without 10 µM quercetin at 39 °C and 5% CO_2. The cleavage rate and the blastocyst rate were determined. Moreover, the intracellular ROS level and mitochondrial activity were determined in the blastocyst derived each experimental groups. Day 7 and 8 blastocysts were frozen at -80 °C until further analysis.

3.3.4. RNA isolation, cDNA synthesis and gene expression analysis

The total RNA was isolated from four biological replicates of each treatment (day 7 and day 8 blastocysts) using Norgen total RNA purification plus kit (Biotek, Canada) according to the manufacturer's instruction. The RNA quality and concentration were measured using a NanoDrop 8000 spectrophotometer (NanoDrop Technologies). Equal concentration of RNA input was reverse transcribed into cDNA using thermo scientific first strand cDNA synthesis kit (Life Technologies, Germany). Briefly, RNA concentration was adjusted using nuclease-free water to total volume 10 µl from each replicate was followed by co-incubation with 0.5 µl oligo (dT)18 and 0.5 µl random primer at 65 °C for 5 min. Next, 1 µl RiboLock, 4 µl 5x reaction buffer, 2 µl dNTPs and 2 µl reverse transcriptase were added for each sample and co-incubated at 25 °C for 5 min, 37 °C for 60 min, and 70 °C for 5 min. After incubation, samples were stored at -20 °C till gene expression analysis.

The relative transcript abundance of Nrf2 and its downstream antioxidant genes, NQO1, PRDX1, SOD1 and CAT were quantified using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany) in Applied Biosystem® StepOnePlus™ (Applied biosystems, Foster City, CA, USA). The 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. The mRNAs expression data was analyzed using the comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schmittgen 2001) and the mean expression value of actin, beta (ACTB) and phosphate dehydrogenase (GAPDH) was used as internal control for normalization. All primers used in this experiment are listed in Table 1 and primers were designed using the NCBI primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

3.3.5. Protein immunofluorescence detection

Immunohistochemistry was performed to quantify and localize the Nrf2 protein in day 7 and day 8 blastocysts. Briefly, blastocysts from each group were washed three times with PBS and fixed in 4 % paraformaldehyde overnight at 4 ^{O}C. Thereafter, fixed samples were washed three times with glycine and permeabilized with 0.5 % triton-X100 (sigma-Aldrich, Munich, Germany) for 3 h with shaking at room temperature. Permeabilized blastocysts were washed three times with PBS and blocked in 4 % donkey serum for 1 h at room temperature followed by overnight incubation at 4 ^{o}C with polyclonal rabbit anti-Nrf2 antibody (1:100, Biorbyt, UK). Thereafter, blastocysts were further incubated with fluorescence-labelled secondary goat anti-rabbit antibody at 37 ^{o}C for 3 hr in the dark (Alexa flour™ 568, 1:350, Life Technologies, Germany). A droplet of Vectashield mounting medium containing (DAPI) (Dabco; Acros, Geel, Belgium) was used to stain the nuclei. Finally, images were visualized under a CLSM LSM-780 confocal lasers scanning microscope (Carl Zeiss GmbH; Germany) and images were analyzed using ImageJ 1.48v (National institutes of Health, USA, http://imagej.nih.gov).

3.3.6. Intracellular ROS accumulation assay

Blastocyst stage embryos were subjected to intracellular ROS level detection using 2´,7´-dichlorofluorescin diacetate (H_2DCFDA) (Life Technologies, Germany) following the manufacture's instruction. Briefly, 15 blastocysts were incubated in 400 µl media containing 5 µM H_2DCFDA for 20 min in dark at 37 ^{O}C, and washed twice in PBS-PVA 0.01 %. Images were captured immediately under fluorescence microscope (Leica DM IRB, Leica, Wetzlar, Germany) using a green-fluorescence filter and analyzed using ImageJ 1.48v (National institutes of Health, USA, http://imagej.nih.gov).

3.3.7. Assessment of mitochondrial activity

The mitochondrial activity was assessed in blastocyst using MitoTracker$^®$ Red CMXRos (M7512; Invitrogen) according to manufacturer's instructions. MitoTracker$^®$ Red CMXRos is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential (MMP), which is a marker for mitochondrial functionality (Poot et al. 2017; Sakamuru et al. 2016). Breifly, lyophilized MitoTracker$^®$ product was dissolved in a high-quality, anhydrous dimethylsulfoxide

(DMSO) to a final concentration of 1 mM. For that, blastocysts were incubated in culture media containing 200 nM MitoTracker® red dye for 45 min, washed twice with PBS-PVA 0.01 %, and fixed overnight at 4 ^{O}C with 4 % paraformaldehyde. Fixed blastocysts were mounted with Vectashield (H-1200) containing DAPI (Dabco; Acros, Geel, Belgium) to stain the nuclei. Images were acquired under a CLSM LSM-780 confocal laser-scanning microscope (Carl Zeiss GmbH; Germany) and analyzed using ImageJ 1.48v (National institutes of Health, USA, http://imagej.nih.gov).

3.3.8. Blastocyst cell number assessment

Total blastocyst cell number was determined using nuclear fluorescence staining (Hoechst 33342,Sigma, Munich; Germany) according to the manufacture's recommendations. Blastocysts from each group were fixed for 5 min in a solution containing 2 % formalin and 0.25 % glutaraldehyde. Morphologically normal appearing embryos (no visible fragmentation, intact zona pellucida and cells) were mounted and stained for 10 min with 12.5 µg/ml glycerol–based Hoechst 33342. Epifluorescent microscope (Carl Zeiss GmbH; Germany) fitted with a blue filter was used to visualize the blue stained nuclei. Finally, images were acquired using Zen blue-edition software. The numbers of cells in each individual blastocyst were counted from each group.

3.3.9. Statistical analysis

Data were analyzed using Graphpad prism 5 (Graphpad, San Diego, CA) and presented as mean±SEM of at least three independent biological replicates. Statistical significance between mean values was determined using one-way analysis of variance (ANOVA) followed by Tukey multiple pairwise comparison was performed between treatment groups. Data from two treatment groups were analysed using two-tailed student's t-test. P values < 0.05 were considered as statistically significant as described in each figure legend.

3.4. Results

3.4.1. In vitro development of bovine preimplantation embryos co-incubated with quercetin

In order to investigate the effect of quercetin on the development of bovine preimplantation embryo development, zygotes were cultured in SOF media supplemented with 10 µM Que till blastocyst stage. Supplementation of quercetin to the culture media showed no significant effect on the cleavage and blastocyst rates (Table 3.2). However, quercetin supplementation in the culture media resulted in significantly increase the day 7 and day 8 blastocyst total cell number compared to the untreated counterpart (Table 3.2).

3.4.2. Quercetin supplementation during in vitro embryo culture reduced intracellular ROS and increased mitochondrial activity

To determine the protective effect of quercetin against oxidative stress in bovine embryo, intracellular ROS accumulation was quantified using H2DCFDA fluorescent reaction (Life Technologies, Germany). Results showed that day 8 blastocysts have higher level of ROS accumulation compared to the day 7 counterpart, irrespective of quercetin supplementation (Fig. 3.1) Moreover, supplementation of quercetin significantly reduced the intracellular ROS level both at day 7 and day 8 blastocysts compared to untreated counterparts (Fig. 3.1), suggesting that quercetin can protects bovine preimplantation embryos against oxidative stress induced by the high oxygen tension (20 %) of the in vitro culture system. Correspondingly, the mitochndirial activity in day 8 blastocyst was lower compared to day 7 blastocysts (Fig. 3.2). Furthermore, quercetin supplementation caused elevation in the mitochondrial activity of day 7 and day 8 blastocysts (Fig. 3.2)

3.4.3. Quercetin modulate embryonic Nrf2 and its downstream antioxidant genes

To obtain further insights into the involvement of quercetin in the protecting of preimplantation embryos against oxidative stress through Nrf2 modulation, the expression level of Nrf2 and its downstream antioxidant genes were quantified. Results showed that quercetin supplementation had no effect on the expression of Nrf2 at day 7 blastocyst stage. However, at day 8 blastocysts stage, a significant increment in the Nrf2 expression was observed (Fig. 3.3). In addition, the expression level of the downstream antioxidants

(NQO1, PRDX1, SOD1 and CAT) was increased at day 7 and day 8 in quercetin treated group compared to the untreated counterparts (Fig. 3.3). On the other hand, the Nrf2 protein level was significantly higher in bovine preimplantation embryos co-incubated with quercetin both at day 7 and day 8 compared to the untreated counterparts (Fig. 3.4).

3.5. Discussion

During in vitro embryo production, exposure of embryos to various environmental stressors affects embryonic development and quality. The suboptimal culture conditions have marked differences in temperature, pH, and oxygen tension compared to the in vivo environment (Guérin et al. 2001; Takenaka et al. 2007; Suzuki and Mittler 2006; Will et al. 2011; Amin et al. 2014). These suboptimal culture conditions are believed to induce oxidative stress conditions as a result of excessive ROS accumulation (Goto et al. 1993). Moreover, the embryo itself is considered as a source of endogenous ROS, generated by the embryonic metabolism (Kang et al. 2009). Oxidative stress results deleterious effect at cellular level such as DNA fragmentation, protein modification and lipid peroxidation (Alvarez 2003; Guérin et al. 2001; Kitagawa et al. 2004; Yu et al. 2014). ROS over accumulation was reported to induce two-cell embryo blockage and may be associated with early embryonic death (Nasr-Esfahani et al. 1990; La Fuente and King 1998). In this regards, our previous results revealed that higher oxygen tension during in vitro embryo culture resulted in higher intracellular ROS accumulation, impaired mitochondrial function (Amin et al. 2014) and the blastocyst transcriptomic profiles differ mainly in the activity of their Nrf2 signaling pathway (Gad et al. 2012; Amin et al. 2014). The Nrf2 is a key transcription factor that regulates a battery of a cytoprotective antioxidant genes against endogenous and environmental stressors (Hayes and Dinkova-Kostova 2014). In our previous work we demonstrated not only the crucial role of Nrf2 in the survival of bovine preimplantation embryos cultured under oxidative stress conditions (Amin et al. 2014) and maintained the function of bovine granulosa cells challenged by oxidative stress condition induced by H_2O_2 (Saeed-Zidane et al. 2017), but also the functional role of Nrf2 in bovine granulosa cells in knockdown and exogenously induced study (Khadrawy et al. 2019). Moreover, supplementation of culture media with antioxidants such as quercetin resulted in reduction of the intracellular ROS level in oocytes and preimplantation embryos (Yu et al. 2014; Sovernigo et al. 2017).

Quercetin is one of the most important flavonoids, which exerts antioxidative capacity in different cell types (Kumar Mishra et al. 2013; Choi et al. 2015) and an anti-inflammatory effect through inhibition of the NF-κB and activation of Nrf2 (Kang et al. 2013a; Saw et al. 2014; Sun et al. 2015). Moreover, in our previous study we demonstrated that quercetin modulate Nrf2-mediated oxidative stress response in bovine granulosa cells through alteration miRNAs expression profile (Khadrawy et al. 2019). However, there is no concrete evidence regarding the effect of quercetin on Nrf2 signaling pathway and the subsequent bovine embryo development and quality. In the present study, bovine preimplantation embryos were cultured in the presence or absence of quercetin to investigate the developmental effect of quercetin on the preimplantation embryos through modulation of Nrf2 signaling pathway. Results demonstrated that supplementation of quercetin at a dose of 10 µM did not significantly affect the cleavage and blastocyst rate (Table 3.2). Contrary to our results, it was reported that quercetin supplementation improves the development of porcine (Kang et al. 2013b; Kang et al. 2016), mouse (Kang et al. 2016) and bovine preimplantation embryos (Sovernigo et al. 2017). Moreover, quercetin supplementation in culture media resulted in increasing total cell number of blastocysts compared to the control group (Table 3.2). These results were in agreement with previous reports, where quercetin supplementation showed an increased total cell number and reduced apoptotic cells in mouse preimplantation embryos (Sameni et al. 2018).

Optimum intracellular ROS level, as a cellular phenotype is associated with embryo development and quality. Many studies demonstrated that accumulation of ROS level above the physiological threshold was correlated with increased embryonic fragmentation and lower cleavage rates (Goto et al. 1993; Bedaiwy et al. 2004). Moreover, competent blastocysts have lower intracellular ROS accumulation compared to their non-competent counterparts (Amin et al. 2014). The reduction in ROS level is reported to be correlated with higher Nrf2 activity and its downstream antioxidant genes in competent early cleavage blastocysts (Amin et al. 2014). In the same context, supplementation of culture media with various antioxidants such as thioredoxin (Bing et al. 2003; Ozawa et al. 2006), glutathione (Ozawa et al. 2006), ascorbic acid (Kere et al. 2013) or L-carnitine (Mishra et al. 2016) resulted in reduction of intracellular ROS in preimplantation embryos and subsequently improved embryo quality. In line to this, the present study illustrated

significant reduction of intracellular ROS level in both day 7 and 8 blastocysts cultured in media supplemented with quercetin (Figure 3.1). Moreover, quercetin supplementation resulted in upregulation of Nrf2 expression level and its downstream antioxidant genes (Figure 3.3 and 3.4). Indeed, quercetin is reported to induce antioxidant genes, such as peroxiredoxins 1 (Khadrawy et al. 2019), peroxiredoxins 3 and 5 (Miyamoto et al. 2011), Heme oxygenase-1 (Sun et al. 2015), SOD1, CAT and NQO1 (Tanigawa et al. 2007; Khadrawy et al. 2019) via the Nrf2 transcription factor.

It is well established that, the most important mitochondrial function is generating ATP. The oocyte developmental competence is associated with adequate mitochondrial functions and the subsequent ATP generation (St John et al. 2010). However, insufficient mitochondrial functions were associated with poor oocyte quality, low embryo development and suboptimal implantation (Wai et al. 2010; Wakefield et al. 2011). Furthermore, Nrf2 signaling pathway is associated with mitochondrial biogenesis, where Nrf2 stimulate the mitochondrial biogenesis via upregulation of nuclear respiratory factor-1 (Nrf1) and antioxidant response (Piantadosi et al. 2008; Merry and Ristow 2016). In the present study, the blastocysts developed from zygotes cultured in quercetin supplemented media, which have higher Nrf2 expression level and lower intracellular ROS accumulation, showed higher mitochondrial activity compared to control (Figure 3.2). The results were in agreement with our previous study in bovine granulosa cells (Khadrawy et al. 2019) and findings from previous studies reporting the protective effects of quercetin on mitochondrial biogenesis and improving mitochondrial dysfunction (Davis et al. 2009; Sandhir and Mehrotra 2013; Li et al. 2016b).

3.6 Conclusion

In conclusion, supplementation of quercetin as exogenous antioxidant into an in vitro embryo culture system under oxidative stress induced by highly oxygen tension was suggested to improve bovine preimplantation embryo quality. Even though quercetin supplementation did not affect cleavage or blastocyst developmental rate, a significant increment in the blastocyst total cell number and reduction of intracellular ROS level accompanied with increased mitochondrial activity was observed. Furthermore, quercetin supplementation activated cellular antioxidant response mechanisms as a result of the activation the Nrf2 transcription factor and its downstream antioxidant genes.
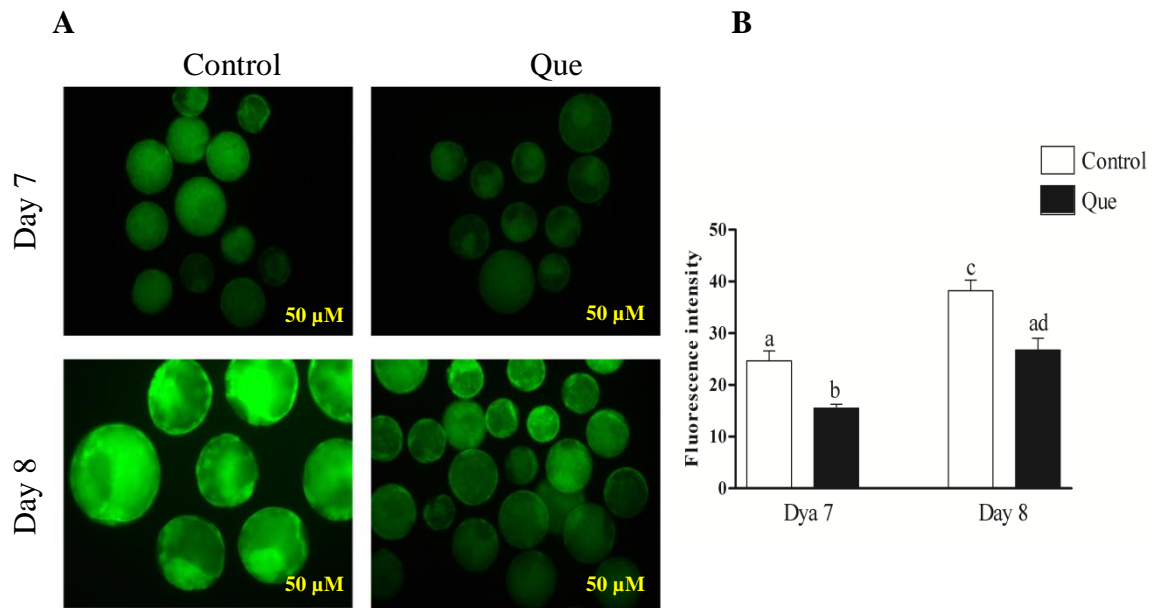
Figure 3.1. Intracellular ROS level in blastocysts developed from zygotes cultured in presence or absence of quercetion (A). The fluorescence intensity analysis of ROS level (B). Control group presented in (white bar) versus quercetin supplemented group (black bar). Data are mean ±SEM of fluorescence intensity from each blastocyst group (a,b,c,d: $p < 0.05$).
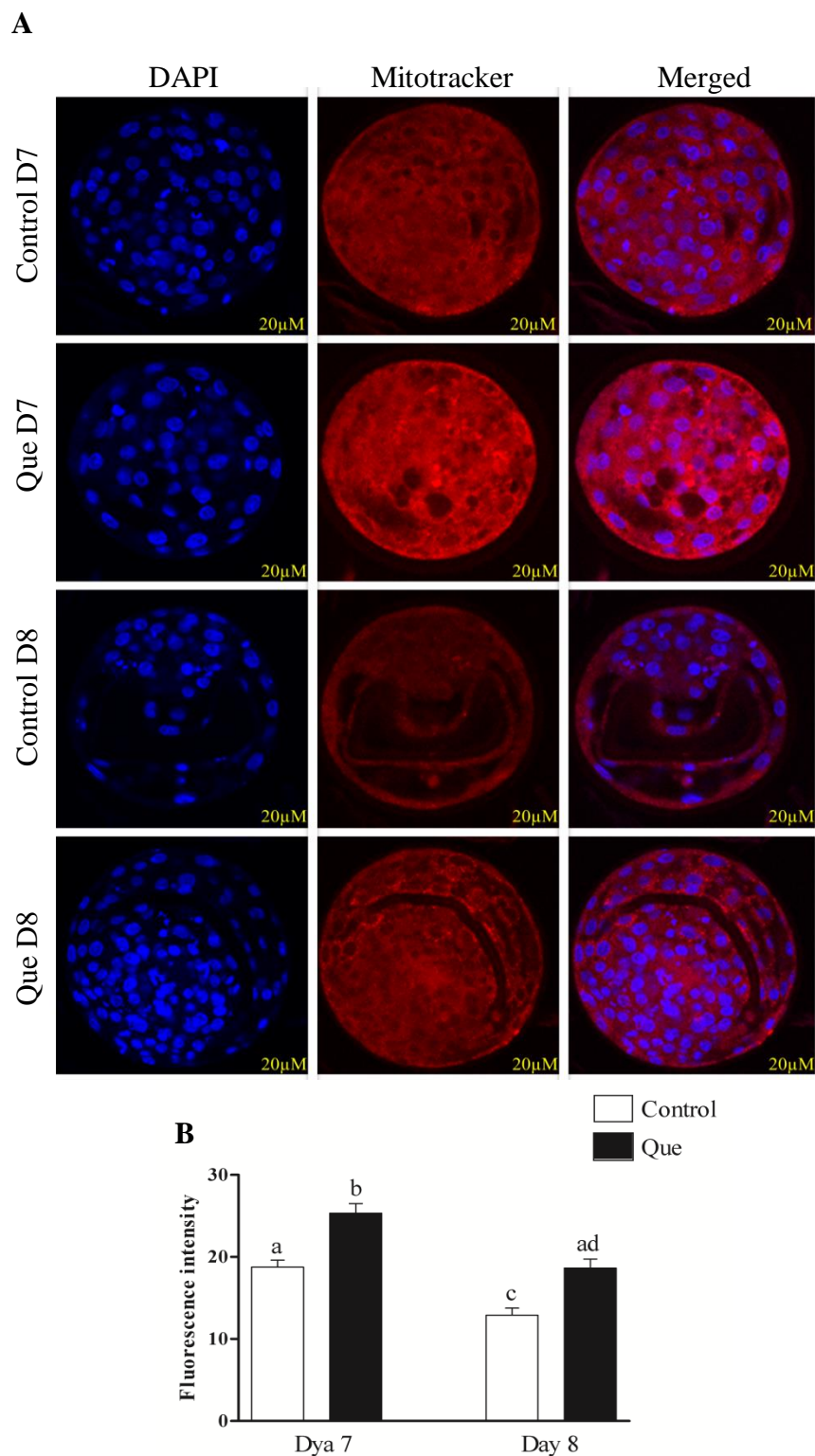
Figure 3.2 Mitochondrial activity in blastocysts developed from zygotes cultured in presence or absence of quercetion (A). The fluorescence intensity analysis of mitochondrial activity (B). Control group presented in (white bar) versus quercetin supplemented group (black bar). Data are mean ±SEM of fluorescence intensity from each blastocyst group (a,b,c,d: $p<0.05$).
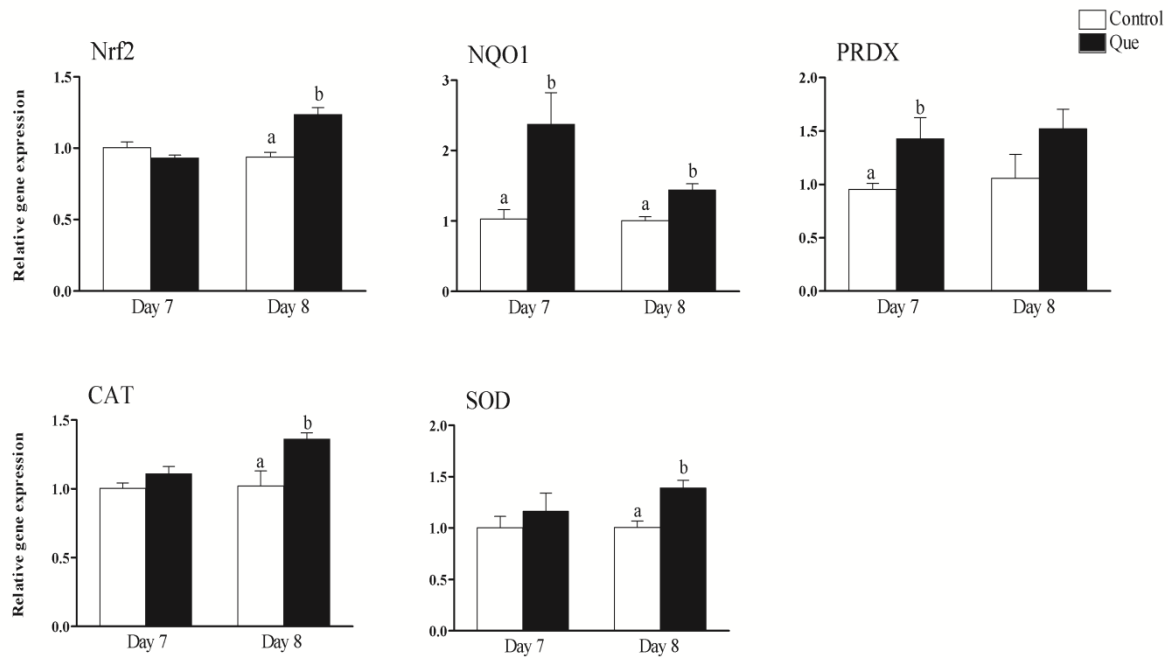
Figure 3.3. Quercetin supplementation modulates Nrf2 and its downstream antioxidant genes in preimplantation embryos. Control group presented in (white bar) versus quercetin supplemented group (black bar). Data are mean ±SEM from four independent biological replicates (a,b: $p < 0.05$).
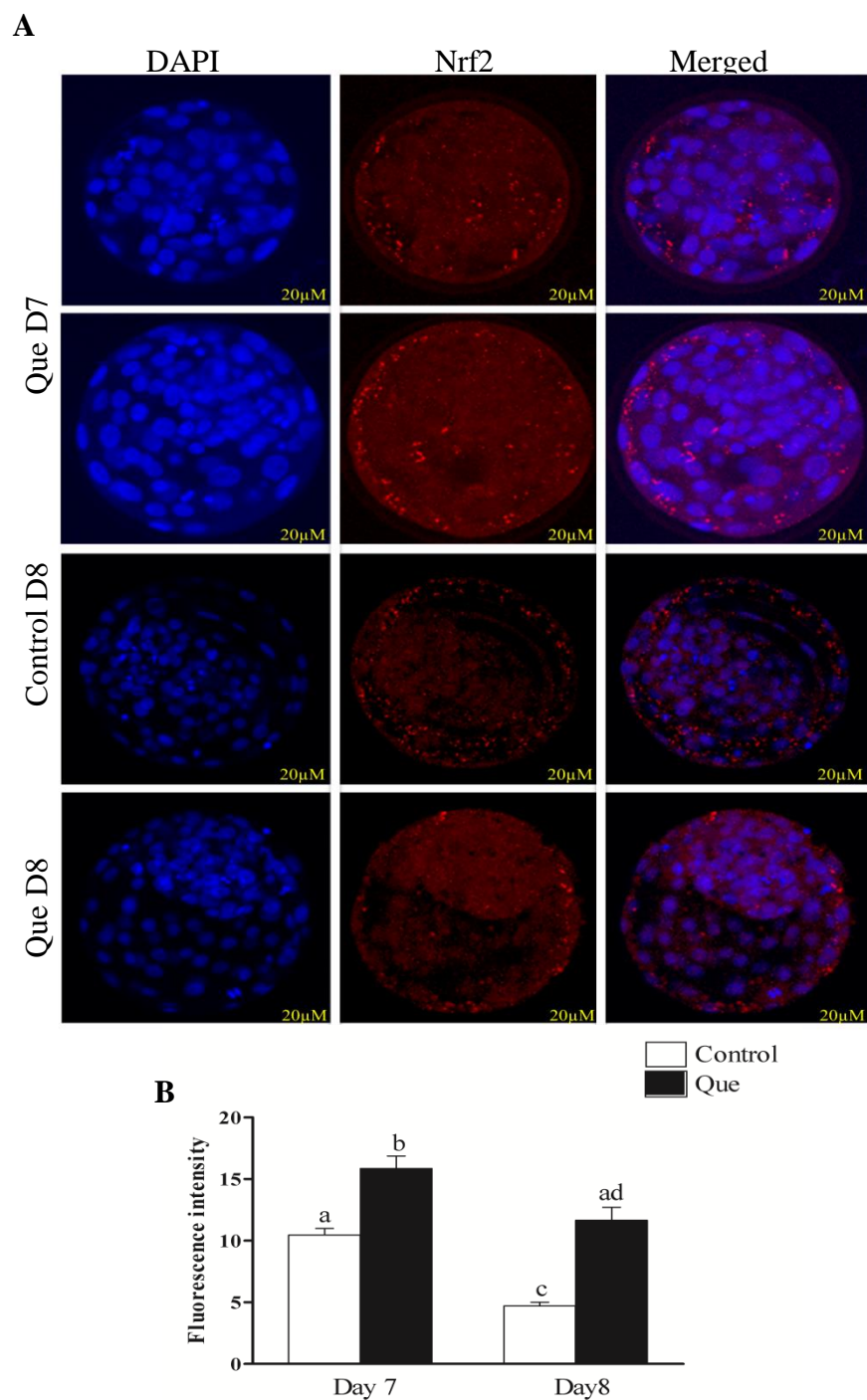
Figure 3.4 Immunocytochemistry of Nrf2 in bovine preimplantation embryos developed from zygotes cultured with or without quercetin supplementation (A). The fluorescence intensity analysis of mitochondrial activity (B). Control group presented in (white bar) versus quercetin supplemented group (black bar). Data are mean ±SEM of fluorescence intensity from each blastocyst group (a,b: p<0.05).

Table 3.1. The list of primers and their sequences of selected candidate genes used for qRT-PCR analysis

Gene	Primer sequence	Size (bp)	Accession number
GAPDH	F: 5´-ACCCAGAAGACTGTGGATGG-3´	247	NM_001034034
	R: 5´-ACGCCTGCTTCACCACCTT-3´		
B-ACTIN	F: 5´-GGCATTCACGAAACTACCTT-3´	208	NM_173979
	R: 5´-CAATCCACACGGAGTACTTG-3´		
Nrf2	F: 5´-CCCAGTCTTCACTGCTCCTC-3´	165	NM_001011678
	R: 5´-TCAGCCAGCTTGTCATTTTG-3´		
NQO1	F: 5´-AACCAACAGACCAGCCAATC-3´	154	NM_001034535.1
	R: 5´-CACAGTGACCTCCCATCCTT-3´		
SOD1	F: 5´-TGCCATCGTGGATATTGTAG-3´	174	NM_174615
	R: 5´-GCAATTCCAATTACACCACA-3´		
PRDX1	F: 5´-TGGATCAACACACCCAAGAA-3´	217	NM_174431.1
	R: 5´-GTCTCAGCGTCTCATCCACA-3´		
CAT	F: 5´-TGGGACCCAACTATCTCCAG-3´	178	NM_001035386.1
	R: 5´-AAGTGGGTCCTGTGTTCCAG-3´		

Table 3.2. The developmental rate and blastocyst total cell number of preimplantation embryos cultured in presence of quercetin

	Control	Quercetin supplemented	P value
No. of zygotes	482	485	
Cleavage rate	83.86 ± 1.27	85.89 ± 1.11	0.20
Day 7 blastocyst rate	27.23 ± 1.68	24.57 ± 4.94	0.64
Day 8 blastocyst rate	38.87 ± 0.85	41.17 ± 3.70	0.58
Day 9 blastocyst rate	42.26 ± 0.97	43.22 ± 3.80	0.82
Day 7 blastocyst total cell number	100 ± 3.74^a	125 ± 7.98^b	0.007
Day 8 blastocyst total cell number	95 ± 3.15^a	128 ± 4.49^b	<0.0001

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Chapter 4

General discussion

4.1 Discussion

Reactive oxygen species (ROS) have double-edged sword in cellular processes. ROS are produced endogenously as by-products through multiple mechanisms such as cellular aerobic respiration via mitochondria and cellular defense mechanism through phagocytic cells (Cantu-Medellin and Kelley 2013; Brand 2016). Under the in vitro embryo culture systems, the suboptimal culture conditions such as oxygen concentration (Orsi and Leese 2001; Booth et al. 2005; Amin et al. 2014), visible light (Umaoka et al. 1992; Goto et al. 1993; Squirrell et al. 1999), spermatozoa (Agarwal et al. 2006) leads to excessive accumulation of intracellular ROS. Optimum amounts of ROS are required for normal physiologic adaptation phenomena (Finkel 2011; Cordeiro et al. 2005; Betteridge 2000). Moreover, ROS are found to be involved in the cellular signaling transduction and mediated the growth factor signaling such as platelet derived growth factor (PDGF) and epidermal growth factor (EGF) (Sundaresan et al. 1995; Bae et al. 1997; Huo et al. 2009; Mesquita et al. 2010). ROS play a crucial role in oocyte maturation (Martín-Romero et al. 2008; Tripathi et al. 2009), ovarian steroid biosynthesis (Fujii et al. 2005) and intermediate decisive changes in cumulus cells prior to ovulation (Shkolnik et al. 2011). Excessive ROS production results in cellular damage due to attacking biomolecules, which are associated with oxidative damage for DNA molecules, protein modifications and lipid peroxidation (Guérin et al. 2001; Kitagawa et al. 2004; Yu et al. 2014; Luo et al. 2006; Alvarez 2003), and furthermore, with impaired oocyte maturation accompanied with poor fertilization and embryo quality (Tarín et al. 1996; Tamura et al. 2008; Bedaiwy et al. 2012). Thus, maintaining redox hemostasis is essential for the development of a competent oocyte and good quality embryos (Liang et al. 2017). The vital role of Nrf2 and its downstream antioxidants for the survival of bovine granulosa cells (Saeed-Zidane et al. 2017) and preimplantation embryos cultured in vitro under oxidative stress conditions has been shown previously. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a member of polyphenolic compounds known as flavonoids. Accumulating evidence demonstrated that quercetin has an antioxidant activity against oxidative stress via the antioxidant machinery in different cell types (Kumar Mishra et al. 2013; Choi et al. 2015) and preimplantation embryos (Yu et al. 2014; Sovernigo et al. 2017).

The Nrf2 was found to be regulated by numerous epigenetic mechanisms such as DNA methylation, histone modifications and miRNAs interactions (Hayes and Dinkova-Kostova

2014; Guo et al. 2015). MicroRNAs are short non-coding RNAs, which were found to be involved in the posttranscriptional regulation of target genes through inhibition of the 3´-untranslated region (3´-UTR) resulting in either reduction of their target mRNA and\or reduced translation of target genes (Bartel 2004). In addition, several cellular processes such as cell proliferation, differentiation, cellular response to different stressors and cellular apoptosis were reported to be regulated by miRNAs (Blenkiron and Miska 2007; Andreas et al. 2016; Gebremedhn et al. 2016; Pande et al. 2018). Thus, in the present study we aimed to investigate the involvement of miRNAs in regulation of the Nrf2 signaling pathway and the subsequent cellular functions in bovine granulosa cells and preimplantation embryos.

In the first experiment, we examined the functional role of candidate miRNAs regulating Nrf2 signaling pathway in bovine granulosa cells. Based on in-silico analysis using online prediction tools (Trgetscan online software, http://www.targetscan.org), three miRNAs (miR-153, miR-28 and miR708) were selected for further analysis. Bovine granulosa cells exposed to oxidative stress induced by H_2O_2, showed increase transcriptional and translational levels of Nrf2 and its downstream antioxidant transcripts (Saeed-Zidane et al. 2017). Previous studies reported that, upregulation of Nrf2 was found to be associated with downregulation of miR-153, miR-93 and miR-28 in different carcinogenic cell models cell lines (Yang et al. 2011; Singh et al. 2013; Wang et al. 2016a). Moreover, oxidative stress conditions resulted in dysregulation of miRNAs expression pattern in cadiomyocytes cells (Yildirim et al. 2013). Here, the candidate miRNAs targeting Nrf2 were analyzed under oxidative stress conditions. Our findings revealed that, the upregulation of Nrf2 in granulosa cells exposed to oxidative stress could be as a result of the reduction in expression pattern of miR-153, miR-28 and miR-708 (Figure 2.1C). The candidate miRNAs, which targeted the 3´-UTR of Nrf2, were validated using Dual-Luciferase® Reporter (DLR™) Assay System (Promega GmbH, Mannheim, Germany) and indicated by the decreased ratio of firefly to Renilla activity in the wild type plasmid variant compared to the mutant counterparts (Figure 2.1B). In order to investigate the functional role of these miRNAs in granulosa cells, cells were transfected with miRNAs mimics and inhibitors. Twenty-four hour later, cells were subjected to phenotypic and genotypic analysis. Findings revealed that ectopic expression of miRNAs resulted in reduction of expression level of Nrf2 and its down stream antioxidant genes. Our results are in

agreement with previous reports, which showed the overexpression effect of miR-153 on Nrf2 mRNA and protein expression in different cell lines (Narasimhan et al. 2012; Yang et al. 2015; Wang et al. 2016a). It is well recognized that Nrf2 signaling pathway and subsequently its downstream antioxidants control the intracellular ROS level (Ma 2013; Kovac et al. 2015). In addition, Nrf2 deficiency resulted in reduction of antioxidant genes accompanied with increase intracellular ROS level (Chen et al. 2015; Gao et al. 2017). Moreover, an increase of intracellular ROS level as a result of H_2O_2 treatment led to the reduction of mitochondrial activity and cellular proliferation rate (Saeed-Zidane et al. 2017). Our results were in agreement with the aforementioned findings. Ectopic expression of miR-153, miR-28 and miR-708 showed an increment in the intracellular ROS level and impaired mitochondrial activity, which caused reduction in the cellular proliferation. Furthermore, we demonstrated the crucial role of Nrf2 in antioxidant machinery under oxidative stress conditions, in which the overexpression of candidate miRNAs under oxidative stress condition showed a reduction in Nrf2 mRNA and protein levels resulted in increasing intracellular ROS level and inadequate mitochondrial distribution, leading to reduced cellular proliferation compared to the H_2O_2 challenged group, suggesting the crucial role of these miRNAs and subsequently Nrf2 signaling pathway in cellular functions specially under oxidative stress conditions.

Nrf2 activators may protect against cellular damage induced by oxidative stress through activating antioxidant machinery systems and reduce ROS production (Yoon et al. 2008; Xu et al. 2016; Wang et al. 2016b). Quercetin is a plant-derived flavonoids mainly polyphenolic compound found in fruits and vegetables has been reported to have an antioxidative effect (Naderi et al. 2003; Kim et al. 2006; Yang et al. 2014; Bao et al. 2017), anti-inflammatory effect (Kang et al. 2013; Saw et al. 2014; Sun et al. 2015) and anti-apoptotic effects (Yang et al. 2014). The present study provides novel information that quercetin confers protection of bovine granulosa cells and preimplantation embryos exposed to oxidative stress conditions via activation of Nrf2 signaling pathway. In the first experiment, we identify the non-toxic dose of quercetin in bovine granulosa cells. Granulosa cells cultured with different concentrations of quercetin for 24 h, followed by phenotype and genotype analysis. Our results showed that quercetin increased the expression of Nrf2 at transcriptional level at dose dependent manner. However, quercetin increases Nrf2 translation only at lower dose 10 µM. Moreover, the 10 µM dose of

quercetin showed improvement in the activity of mitochondria and cellular proliferation. However, higher doses (20-200 µM) showed increasing intracellular ROS and reduced mitochondrial activity in dose-dependent manner (Figure 2.S3). Several studies have revealed the antioxidant properties of quercetin in cells (Kumar Mishra et al. 2013; Choi et al. 2015) and preimplantation embryos subjected to oxidative stress (Yu et al. 2014; Sovernigo et al. 2017). However, quercetin has the capability to act as prooxidant depending on the concentration supplemented (Robledinos-Antón et al. 2017), where lower concentration could have an antioxidant effect, while higher concentrations could cause cellular damage indicated by reduction of cellular proliferation followed by apoptosis (Robaszkiewicz et al. 2007), which stimulate the generation of superoxide radicals (O_2-) and subsequently affecting mitochondrial activity (Marchi et al. 2009). Thus, in the present study quercetin at low dose (10 µM) was selected to study its protective effect against oxidative stress conditions in granulosa cells and preimplantation embryos.

Bovine granulosa cells exposed to oxidative stress induced by H_2O_2 were treated with 10 µM of quercetin. A significant reduction in intracellular ROS level accompanied with improvement of the cellular proliferation and mitochondrial activity, which is associated with Nrf2 activation and the subsequent downstream antioxidant genes. Likewise, quercetin exerts a protective effect on preimplantation embryos cultured at high atmospheric oxygen tension (20 %). Our results showed that, quercetin modulates the Nrf2 signaling pathway and the cellular antioxidant machinery (Figure 3.3). Moreover, quercetin supplementation in embryo culture media resulted in reduction of the intracellular ROS level, increasing blastocyst total cell number and mitochondrial activity. Our results are in agreement with previous studies, which have discussed the protective effect of quercetin against oxidative stress conditions (Zhao et al. 2014; Wang et al. 2014; Bao et al. 2017). Several lines of studies revealed that phytochemicals-mediated miRNAs expression in cancer cells (Srivastava et al. 2015) and investigated the impact of flavonoids on miRNAs expression in vivo and in vitro (Chakrabarti et al. 2012; Tsang and Kwok 2010; Milenkovic et al. 2012; Chiyomaru et al. 2012; Parker et al. 2009; Sun et al. 2008; Mukhopadhyay et al. 2010). Among flavonoids, quercetin, which altered the expression pattern of miRNAs (Milenkovic et al. 2012; Boesch-Saadatmandi et al. 2011; Boesch-Saadatmandi et al. 2012; Zhang et al. 2015; Del Follo-Martinez et al. 2013; Chuammitri et al. 2017). Here, in the present study our results revealed for the first time the exogenous

modulation of Nrf2 and its downstream antioxidant genes induced by quercetin in bovine granulosa cells could be mediated by miRNAs (miR-153, miR-28 and miR-708) (Figure 2.9).

4.2 Conclusion and future prospective

The present study demonstrated the potential role of miRNAs (miR-153, miR-28 and miR-708) in the regulation of Nrf2 signaling pathway and the subsequent bovine granulosa cell functions. Moreover, increase Nrf2 expression pattern in bovine granulosa cells exposed to oxidative stress condition or co-incubated with quercetin as Nrf2 activator was accompanied with the reduction in the expression of miR-153, miR-28 and miR-708. Furthermore, Nrf2 knockdown using siRNAs to cross validate the effect of overexpression of miR-153 and miR-28/708 on bovine granulosa cell functions. On the other hand, quercetin supplementation in culture media protects granulosa cells and preimplantation embryos from oxidative stress conditions.

Further investigations are needed to understand the Nrf2 regulatory network during oocyte and embryo development. Therefore, future experiment could be directed towards investigating:

– Cellular response via Nrf2 signaling pathway against different cellular stressors (heat stress, cold stress, inflammatory stress, etc.)

– The potential role of miRNAs regulating NRF2 mediated oxidative stress response in oocyte maturation and early embryo development

– Correlation between Nrf2 gene methylation pattern and oxidative stress condition in granulosa cells and preimplantation embryos

– Functional role of Nrf2 signaling pathway in oocyte and preimplantation embryos using CRISPR gene editing system

– Detection SNPs on Nrf2 gene and variant dependent activity of NRF2 during oxidative stress in oocytes and embryos.

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6. Research publication

Peer reviewed published articles

1. Ahmed E. Ahmed, Alaa Eldin Zain Elabdeen, & **Omar Zain Khadrawy** (2017). Presynchronization with CIDR improved the efficiency of ovsynch in the cyclic and acyclic Postpartum Pluriparous Cows. Zagazig Veterinary Journal, 45, 82-91.

2. **Omar Khadrawy**, Samuel Gebremedhn, Dessie Salilew-Wondim, Mohamed Omar Taqi, Christiane Neuhoff, Ernst Tholen, Michael Hoelker, Karl Schellander and Dawit Tesfaye (2019). Endogenous and Exogenous Modulation of Nrf2 Mediated Oxidative Stress Response in Bovine Granulosa Cells: Potential Implication for Ovarian Function. Int. J. Mol. Sci. *20*, 1635.

3. Mohamed Omar Taqi, Mohammed Saeed-Zidane, Samuel Gebremedhn, Dessie SalilewWondim, **Omar Khadrawy**, Franca Rings, Christiane Neuhoff, Michael Hoelker, Karl Schellander and Dawit Tesfaye (2019). Sexual dimorphic expression and release of transcription factors in bovine embryos exposed to oxidative stress. Mol Reprod Dev. 2019;1–15.

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1. Quercetin modulates Nrf2-mediated oxidative stress response in bovine preimplantation embryos. **Omar Khadrawy**, Samuel Gebremedhn, Dessie Salilew-Wondim, Mohamed Omar Taqi, Franca Rings, Christiane Neuhoff, Ernst Tholen, Eva Held, Michael Hoelker, Karl Schellander and Dawit Tesfaye (under submission)

Conference abstracts

1. **O. Khadrawy**, S. Gebremedhn, D. Salilew-Wondim, C. Neuhoff, E.Tholen, E. Held, M. Hoelker, K. Schellander, D. Tesfaye (2017). Endogenous and exogenous modulation of Nrf2-mediated oxidative stress response in bovine granulosa cells. Vortragstagung der DGfZ und GfT, 20/21.9.2017, Stuttgart, Germany, (Abstr, oral presentation).

2. **O. Khadrawy**, S. Gebremedhn, D. Salilew-Wondim, F. Rings, E. Held, M. Hölker, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye (2018). Exogenous modulation of Nrf2-

mediated oxidative stress response in bovine preimplantation embryos. Vortragstagung der DGfZ und GfT am 12./13. 9. 2018, Bonn, Germany, (Abstr, oral presentation).

3. **O. Khadrawy**, S. Gebremedhn, D. Salilew-Wondim, F. Rings, C. Neuhoff, E. Tholen, E. Held-Hoelker, M. Hoelker, K. Schellander and D. Tesfaye (2019). Quercetin protects bovine pre-implantation embryos against oxidative stress via activation of Nrf2 signaling pathway. 20-23.1.2019), IETS conference, New Orleans, Louisiana, USA (Abstract, Poster).