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NRF2 mediated oxidative stress response activity during early in vitro bovine embryo development

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Dedicated to my family

NRF2 mediated oxidative stress response activity during early in vitro bovine embryo development

Overcoming oxidative stress is one of the various embryo challenges to survive under suboptimal conditions during in vitro production of bovine embryos. Thus, the present study aimed to examine the ability of preimplantation bovine embryos to activate nuclear factor erythroid-derived 2-like 2 (NFE2L2 or NRF2)-mediated oxidative stress response and trigger their survival under oxidative stress conditions. An in vitro model was used to culture embryos under low (5%) oxygen tension as in bovine oviduct or high oxygen tension (20%), which is widely used in vitro culture of embryos. Early stage embryos including 2-, 4-, 8-, 16-cell and blastocyst stage embryos were generated under low (5%) or high (20%) oxygen level culture conditions. NRF2, NRF2 cytoplasmic inhibitor (KEAP1) and selected NRF2 target antioxidant genes expression were measured in each stage using quantitative real time PCR (qPCR). Reactive oxygen species (ROS) were evaluated in the blastocysts using green fluorescent probe. Our results revealed that the ROS level was high under 20 % compared to 5 % oxygen level in blastocysts. The transcription level of NRF2 and its downstream antioxidant genes was dramatically increased in 8-, 16-cell and blastocyst stage embryos under high compared to low oxygen level, while NRF2 inhibitor showed opposite expression pattern. In order to know whether NRF2 activity is associated with the embryo developmental competence, consequently NRF2 activity was compared in developmentally competent versus incompetent embryos. For this, the mRNA and protein expressions of NRF2 and the transcription level of its downstream antioxidant genes were compared in early (competent) vs. late (incompetent) cleaving 2-cell and blastocyst stage embryos. In the early developing blastocysts accompanied by low ROS level, NRF2 and its antioxidant target genes expression were increased. Likewise, protein expression pattern was observed in similar manner with more active nuclear NRF2. In conclusion, this study demonstrated that under oxidative stress conditions, pre-implantation bovine embryos are able to activate the NRF2-mediated oxidative stress response pathway, which is found to be correlated with their survival under in vitro condition.

NRF2 vermittelte oxidative Stressreaktion während der frühen bovinen in vitro Embryoentwicklung

Für bovine Embryonen ist das Überwinden von oxidativem Stress eine wichtige Herausforderung um in suboptimalen in vitro Entwicklungsbedingungen überleben zu können. Das Ziel dieser Studie war es, die Reaktionsfähigkeit und Überlebensfähigkeit von pre-implantierten bovinen Embryonen auf den durch den nuclear factor erythroid-derived 2-like 2 (NFE2L2 oder NRF2)-vermittelten oxidativen Stress zu untersuchen. Für diese Studie wurde ein in vitro Kulturmodell mit unterschiedlicher Sauerstoffkonzentration (5%, 20%) etabliert. Dabei ähnelte die niedrige Sauerstofftension (5%) der im bovinen Eileiter und die höhere (20%) der die normalerweise zur in vitro Kultur von Embryonen benutzt wird. Frühe embryonal Stadien, 2-, 4-, 8-, 16-Zell- und Blastozystenstadien wurden unter geringen (5%) oder hohen (20%) Sauerstoffkonzentrationen kultiviert. Anschließend wurden die Genexpressionen von NRF2, NRF2 cytoplasmic inhibitor (KEAP1) und ausgewählten NRF2 Antioxidans-Zielgenen in den verschieden Stadien mittels quantitative real time PCR (qPCR) analysiert. Reactive oxygen species (ROS) wurden in Blastozyten mittels green fluorescent probe untersucht. Das Ergebnis zeigte, dass der ROS Spiegel in Blastozysten bei einer Sauerstofftension von 20% höher war im Vergleich zu der 5% Gruppe. Die Expression von NRF2 und seinen nachgeschalteten Antioxidans-Genen war unter einem hohen Sauerstoffspiegel im Vergleich zu einem niedrigeren in 8-, 16-Zell- und Blastozytenstadien dramatisch erhöht. Demgegenüber zeigte NRF2 Inhibition ein gegenteiliges Expressionsmuster. Um festzustellen, ob die NRF2 Aktivität mit der Embryoentwicklungsfähigkeit zusammen hängt, wurde die NRF2 Aktivität im Vergleich von entwicklungsfähigen zu nicht entwicklungsfähigen Embryonen untersucht. Dafür wurden die NRF2 mRNA- und Proteinexpressionen und die der Antioxidans-Gene im Vergleich von früh (kompetent) zu spät (inkompetent) entwickelten 2-Zell- und Blastozytenstadien analysiert. Die Genexpression von NRF2 und deren Antioxidans-Zielgenen war in früh entwickelten Blastozyten bei einem geringen ROS Spiegel erhöht. Ein ähnliches Bild zeigte sich für die Proteinexpression mit einem größeren aktiven Anteil an nuklearem NRF2. Schlussendlich zeigte diese Studie, dass unter oxidativen Stress pre-implantiere bovine Embryonen in der Lage sind den NRF2-vermittelten oxidativen Stressreaktionssignalweg zu aktivieren. Dieser steht in Korrelation zu der Überlebensfähigkeit unter in vitro Bedingungen.

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List of abbreviations

$^1O_2$	Singlet oxygen
ACACA1	Acetyl-CoA carboxylase alpha
AIF	Apoptosis inducing factor
AREs	Antioxidant response elements
ATP	Adenosine triphosphate
Bax	Bcl2-associated X protein
CAT	Catalase
COCs	Cumulus oocyte complexes
CPT2	Carnitine palmitoyl transferase-2
CR1-aa	Charles Rosenkrans medium supplemented with amino acids
CSH	Cysteamine
CUL3	Cullin 3
D3T	3H-1,2 dithiole-3-thione
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
E3	Ubiquitin conjugating enzyme
EB	Early blastocyst
EC-2C	Early cleaving 2-cell
EDTA	Ethylenediamine tetra-acetic acid
EPS	Excited photosensitizers
ER	Endoplasmic reticulum
ERO-1	Endoplasmic reticulum membrane associated oxidoreductin1
ET	Electron transfer
ETC	Electron transport chain
FADD	Fas-associated death domain containing protein
FITC	Fluorescein isothiocyanate
G6PD	Glucose-6-phosphate dehydrogenase
GCLC	Glutamate–cysteine ligase catalytic subunit

GCLM	Glutamate–cysteine ligase complex modifier subunit
GPBS	Glycin in PBS
GPX	Glutathione peroxidase
GSH	Glutathione
GSH-OEt	Glutathione reduced ethyl ester
GSR	Glutathione reductase
GSTA1	Glutathione S-transferase alpha 1
GSTA2	Glutathione S-transferase alpha 2
GSTA3	Glutathione S-transferase alpha 3
GSTA5	Glutathione S-transferase alpha 5
GSTM1	Glutathione S-transferase mu 1
GSTM2	Glutathione S-transferase mu 2
GSTM3	Glutathione S-transferase mu 3
GSTP1	Glutathione S-transferase pi 1
GSTs	Glutathione S-transferase family
H2DCFDA	6-carboxy- 2',7'-dichlorodihydrofluorescin diacetate
H_2O_2	Hydrogen peroxide
HEPA	High efficiency particulate air
HMOX1	Heme oxygenase 1
HO_2•	Hydroperoxyle
$HOCl^-$	Hypochlorous acid
HPRT	Hypoxanthine phosphoribosyl transferase
ICM	Inner cell mass
IDH1	Isocitrate dehydrogenase 1
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
KEAP1	Kelch-like ECH associated protein 1
LB	Luria Bertani
LB	Late blastocyst
LB-agar	Luria broth-agar
LC-2C	Late cleaving 2-cell

ME1	Malic enzyme 1
mRNA	Messenger ribonucleic acid
mtDNA	mitochondrial DNA
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
Neh	NRF2-ECH homology
NES	Nuclear export sequence
NLE	Neem leaf extract
NO	Nitric oxide
Nox4	NADPH oxidase 4
NQO1	NAD(P)H dehydrogenase quinone 1
NRF2	Nuclear factor erythroid-derived 2-like 2
O_2•-	Superoxide
O_3	Ozone
OCS	Oestrus cow serum
OH•	Hydroxyl radical
ONOO-	Peroxynitrite
OPU	Ovum pickup
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PFA	Para formaldehyde
PHGDH	Phosphoglycerate dehydrogenase
pi	Post insemination
PPARAα	Peroxisome proliferator-activated receptor alpha
PRDX1	Peroxiredoxin 1
PS	Photosensitizers
qPCR	Quantitative real time PCR
RBX1	Ring box 1
RO•	Alkoxyl
RO_2•	Peroxyl
ROS	Reactive oxygen species

Sirt3	Sirtuin 3
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
SODs	Superoxide dismutase family
SREBP1	Sterol regulatory element binding protein transcription factor1
TAE	Tris-acetate-EDTA buffer
TCA	Tricarboxylic acid cycle
TE	Trophoblast
TE	Tris-EDTA
TNF	Tumour necrosis factor
TXN	Thioredoxin
TXN1	Thioredoxin 1
TXN2	Thioredoxin 2
TXNRD1	Thioredoxin reductase 1
VOCs	Volatile organic compounds
XCT	Cysteine/glutamate transporter
β-ME	Beta-mercaptoethanol

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1 Introduction

Despite significant developments achieved in in vitro production of mammalian embryos in the last decade, in vitro produced (IVP) mammalian embryos differ from various factors between the two environments including media components, pH and oxygen level. These environmental conditions, which are still suboptimal, are supposed to be the sources of stress for IVP embryos as manifested by the accumulation of reactive oxygen species (ROS) (Goto et al. 1993). The imbalance between concentration of intracellular ROS and the ROS scavenging ability of cell through antioxidants leads to the state of oxidative stress (Agarwal et al. 2003, Agarwal et al. 2005b, Van Guilder et al. 2006). Oxidative stress is mediated by ROS such as superoxide (O_2•-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), which are not only exerted from the environment but also generated internally in oocytes and embryos as by-product of the energy metabolism, specially, during mitochondrial phosphorylation and glycolysis (Balaban et al. 2005, Guerin et al. 2001). ROS are highly reactive molecules that can interact and damage other cell molecules such as DNA (Mello Filho et al. 1984), proteins and lipids (Wu and Cederbaum 2003), cell structures such as cell membrane due to lipid peroxidation and thereby impair mitochondrial integrity and activity (Kadenbach et al. 2004, Powers and Jackson 2008). Therefore, high oxidative stress under in vitro conditions can negatively affect oocyte maturation and early embryo development by inducing apoptosis thereby embryo fragmentation (Bedaiwy et al. 2004, Du and Wales 1993, Johnson and Nasr-Esfahani 1994, Khurana and Niemann 2000, Liu and Keefe 2000), changes in gene expression pattern and embryonic metabolism (Balasubramanian et al. 2007, Du and Wales 1993, Harvey et al. 2007, Rinaudo et al. 2006), lipid accumulation and reduced embryo quality (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007a, Sudano et al. 2011). Thus, embryo protection mechanisms against oxidative stress are one of the key elements in improving embryo quality thereby influencing embryo developmental competence under in vitro condition (Takahashi 2012). In the last decade the involvement of different compounds in the cellular defense mechanisms against oxidative stress has been elucidated by various reports. Among these, non-enzymatic compounds such as vitamin A, C and E (Pascoe et al. 1987, Schweigert and Zucker 1988), pyruvate (Morales et al. 1999), cysteine (Ali et al. 2003), glutathione (GSH) and glutathione ethyl ester (GSH-OEt) (Curnow et al. 2010, Luvoni et al. 1996, Takahashi et al. 1993) have been used as exogenous inputs

into the culture media to improve embryo development. In addition, antioxidant enzymes namely: glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD) and thioredoxin (TXN) have been shown to be involved in ROS scavenging to protect embryos from oxidative stress (Abedelahi et al. 2010, Li et al. 1993, Natsuyama et al. 1993, Nonogaki et al. 1991, Nonogaki et al. 1992, Ozawa et al. 2006). Recently, series of experiments have shown the stage specific effect of alternative culture of bovine embryos on the transcriptome profile of the resulting blastocysts (Gad et al. 2012). In that study, functional classification and pathway analyses of those genes affected by alternative culture environment revealed that NRF2-mediated oxidative stress response pathway to be the dominant pathway. In NRF2-antioxidant signaling pathway, the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) namely, NRF2 is a key transcriptional factor which regulates many antioxidant genes including catalase (CAT), heme oxygenase (decycling) 1 (HMOX1), NAD(P)H dehydrogenase quinone 1 (NQO1), peroxiredoxin 1 (PRDX1), superoxide dismutase 1 (SOD1) and thioredoxin 1 (TXN1) via the NRF2-KEAP1 cytoplasmic oxidative stress system in mammalian cells (Nguyen et al. 2003, Nguyen et al. 2009, Tanaka et al. 2008, Wild et al. 1999). Kelch-like ECH associated protein 1 (KEAP1) is known to play a regulatory role of NRF2 (Zhang 2006). In agreement with the report that suggested the role of NRF2 in the lipid metabolism as well as lipid accumulation in mouse liver (Huang et al. 2010, Okada et al. 2009, Tanaka et al. 2008), significant number of genes associated with lipid metabolism were also found to be affected due to alternative culture of embryos in vitro or in vivo. As the effect of alternative culture environment on the resulting embryos has been investigated only in those embryos which have survived and reached to the blastocyst stage (Gad et al. 2012), it was not possible to elucidate the oxidative stress defense mechanism through NRF2 activity in those embryos which did not survive and reached to the blastocyst stage. Therefore, it has been hypothesized that embryos which survive and reached to the required stage of development were able to cope up with unfavorable conditions in in vitro culture as a result of their ability to activate their defence mechanism in form of NRF2 mediated oxidative stress response to prevent the accumulation of ROS. Looking into differences between in vitro and in vivo environments, in addition to culture ingredients, the two environments differ in their oxygen level, which is 20% in vitro and 5-7% in vivo. The high level of oxygen in in vitro culture can be one of the sources of oxidative stress for embryos. Therefore, in the

present study, low (5%) and high (20%) oxygen levels were used in in vitro culture media condition to investigate the effect of oxidative stress on embryo development and activity of NRF2 mediated oxidative stress response pathway and its association with embryo survival and metabolism.

2 Literature review

Reproduction is the backbone of dairy and beef industry. Implementation of new reproductive techniques in cattle such as superovulation, non-surgical oocyte and embryo recovery, oocytes and embryos cryopreservation, embryo transfer and in vitro production (IVP) of embryos provided a promising new tool for cattle breeding. Production of huge numbers of calves from cows of superior genetic merit by using these techniques offers an increase in the selection intensity and facilitates a shortening of the generation interval as well as improves the genetic gain during short time. Not surprisingly, these new embryo techniques were rapidly accepted by the cattle breeding industry (Merton et al. 2003).

2.1 In vitro production of embryos

IVP of cattle embryos has been around for about 30 years and improved basically for potential breeding objectives. Lu et al. (1987) created the first calf from total in vitro procedures. Over the years, it has been considered as a one of the most important and applicable reproductive techniques to improve the reproductive capacity of the superior animals, as consequence, increase the genetic improvement rate as well as the productivity of dairy and beef cattle breeds. In addition, IVP of embryos using oocytes either from live cows by using ovum pickup (OPU) technique or from ovaries of commercially slaughtered cows has provided an important and intensive source of embryos for basic research purposes (Betteridge 2003, Bols et al. 2012, Hyttel et al. 2000, Merton et al. 2003). IVP of embryo process basically includes three essential steps (Lu et al. 1987), as shown in Figure 1. The first step is the in vitro maturation (IVM) of the oocyte. Oocyte maturation process is defined as a lengthy process, during which the oocyte conquers the competence to be fertilized and go through embryogenesis. During IVM process, oocyte resumes and completes meiosis, developing from prophase of the first meiotic division to metaphase II (MII). As consequence, the primary oocyte is transformed into a mature secondary oocyte, which is accompanied by a series of changes in the oocyte nucleus and the cytoplasm. The induction of IVM of bovine oocyte is usually performed by incubation of oocyte with specific complex maturation medium for 24 hours (Fukui et al. 1991, Fulka et al. 1995, Hardy et al. 2000, Tomek et al. 2002). The second step in the IVP procedure is the in

vitro fertilization (IVF) of the resulted in vitro matured oocyte. IVF is a process by which an oocyte is fertilized in vitro by sperm. IVF of bovine oocyte is performed by incubation of the matured oocyte for 24 hours either with fresh or frozen–thawed treated semen which allows the greatest flexibility to select the paternal genetic material (Fukui et al. 1991, Leibfried-Rutledge et al. 1989). The third step in the IVP procedure is the in vitro culture (IVC) of the resulted embryo after IVF of oocyte. The fertilized oocyte is developed to be a zygote wherein the nuclei of the oocyte and the sperm, which are haploid nuclei, exist together as separate pronuclei before remodelling to form a diploid genome. The first cleavage following fertilization occurs within 28-35 hours post insemination (pi) (Holm et al. 1998). Cleavages are a chain of mitotic divisions without cell growth by which the massive volume of oocyte cytoplasm splits into several smaller nucleated cells (Bazer et al. 1987). Additional rounds of embryo cleavages following first cleavage occurs and the embryo reaches the 4-cell stage within 40-48 hours pi, the 8-cell stage by 52-86 hours pi, the 16-cell stage by 90-116 hours pi, the morula stage at day 5 and blastocyst stage within 7-9 days (Grisart et al. 1994, Holm and Callesen 1998, Holm et al. 1998). At these early stages of development "from zygote to blastocyst formation", the bovine embryo is a vastly dynamic and major developmental events take place during this period. These events include the first cleavage division, which is considered as an early indicator for the embryo developmental competence (Bernardi and Delouis 1996, Grisart et al. 1994, Lonergan et al. 1999, Plante et al. 1994, Van Soom et al. 1992), in addition, the embryonic genome activation at the 8–16-cell stage, the morula compaction at day 5 and differentiation of compacted morula cells into two types: an inner cell mass (ICM) and trophoblast cells (TE), as a result, the blastocyst formation at day seven (Betteridge and Flechon 1988). Under in vitro environment, all these events are affected by the in vitro culture conditions and affect the resultant embryo characteristics, quality and developmental competence, consequently resulting in poor embryo developmental rates to blastocyst (30–40%) (Gutierrez-Adan et al. 2001, Niemann and Wrenzycki 2000) and low pregnancy rates after transfer into recipients (Lonergan et al. 2003a, Lonergan et al. 2007).

Bovine IVP embryos have several characteristics that are different from the in vivo developed embryos, including gene expression (Corcoran et al. 2006, Gad et al. 2012), ultrastructure (Crosier et al. 2000, Rizos et al. 2002a), lipid content (Sudano et al.

2012), chromosomal abnormalities (Viuff et al. 1999), DNA methylation patterns (Niemann et al. 2010) and cryotolerance (Rizos et al. 2003).

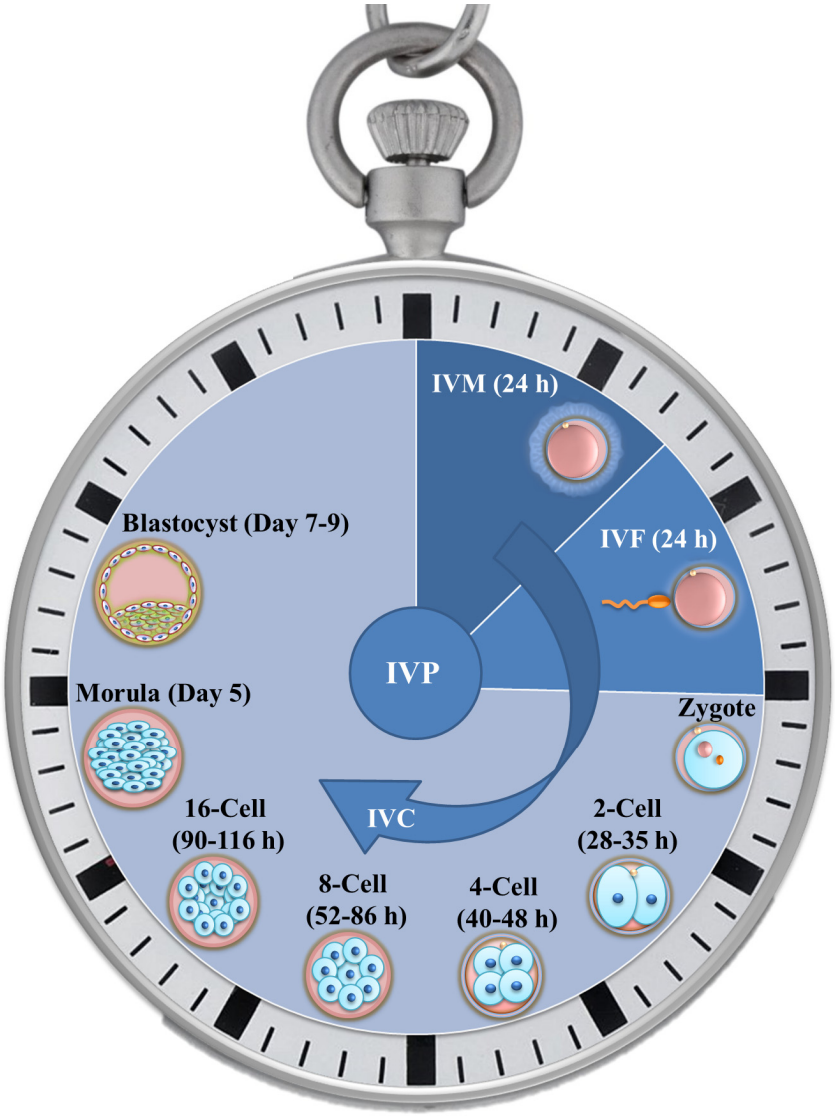


Figure 1: Essential steps of IVP of bovine embryos including IVM and IVF of oocyte and IVC of resultant embryo from zygote to blastocyst stage of development.

Furthermore, it has been known for more than a decade that production of mammalian embryos in vitro is associated with developmental abnormalities in resultant fetuses and offspring (Farin et al. 2006). These abnormalities include increased rates of early embryonic death and abortion, production of large size fetus and offspring “called large offspring syndrome”, musculoskeletal, organ growth and fetal growth abnormalities

(Behboodi et al. 1995, Farin and Farin 1995, Farin et al. 2001, Kruip and denDaas 1997, Sangild et al. 2000, Wakschlag et al. 2007). One of the major factors by which the in vitro environment can affect the embryo quality and developmental competence during the preimplantation period is through the induction of oxidative stress (Abe et al. 2002, Balasubramanian et al. 2007, Barcelo-Fimbres and Seidel 2007a, Du and Wales 1993, Harvey et al. 2007, Johnson and Nasr-Esfahani 1994, Khurana and Wales 1989, Khurana and Niemann 2000, Liu and Keefe 2000, Rinaudo et al. 2006, Sudano et al. 2011).

2.2 Oxidative stress

Oxidative stress is a disturbance in the balance between the production of ROS and the availability and activity of antioxidant defense mechanisms that are responsible for scavenging the ROS in the cell (Basu 2010, Betteridge 2000, Sies 1986). Therefore, oxidative stress can result either from exposure to increased level of oxidants or from lack of defense against oxidants, or from both of them (Davies 2000). So, cells can tackle the oxidative stress by reducing ROS production or by inducing the activity and availability of the antioxidants (Agarwal et al. 2005b). Under oxidative stress conditions, ROS can attack and damage the other important biological molecules such as DNA, proteins and lipids. On the other hand, under stress free conditions, ROS has a beneficial role in some physiological processes and in the intracellular signal transduction regulation (Mittler et al. 2011).

In the last few decades, oxidative stress has been one of the focal points amongst the researchers in the field of reproduction all over the world. Several factors contribute to this attraction to the research including: 1) increasing the knowledge about reactive oxygen production, structure, functions and metabolism, 2) identification of biomarkers for oxidative damage, 3) association of reproductive disorders in terms of infertility and oxidative stress, 4) ample evidences on the harmful effect of oxidative stress on the maturation and fertilization of oocyte and early embryo development during in vitro production of embryos, 5) identification of many antioxidants that can be supplemented to the culture media to assist protection against oxidative stress (Agarwal et al. 2012, Cetica et al. 1999, Cetica et al. 2001, de Matos and Furnus 2000, de Matos et al. 2002, Iudica et al. 1999, Rahal et al. 2014).

2.2.1 Reactive oxygen species (ROS)

Oxidative stress is mediated by ROS, which include a variety of molecules generated from molecular oxygen including free radicals ,such as superoxide (O_2•-), hydroxyl radical (OH•), peroxyl (RO_2•), alkoxyl (RO•) and hydroperoxyl (HO_2•), and non-free radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl^-$), ozone (O_3) and singlet oxygen ($^1O_2$) (Covarrubias et al. 2008, Sies 1986). ROS are highly reactive molecules which have at least one unpaired electron in the atom's outer shell. Thus, they are more active than the oxygen molecule and have more ability to interact and damage other cellular molecules and structures (Kadenbach et al. 2004, Mello Filho et al. 1984, Wu and Cederbaum 2003). The most commonly known ROS in the biological systems are O_2•-, H_2O_2, OH• and $^1O_2$. Generally, they are generated as byproducts during the physiological process involving oxygen consumption (Fujii et al. 2005). In addition ROS can also interact with the other reactive species, such as reactive nitrogen species (i.e. nitric oxide (NO), to generate multiple reactive species or hybrid reactive species "hybrid RS", for instance, peroxynitrite (ONOO-) can be produced from the chemical interaction between NO and O_2•-. Moreover, other chemical interactions can convert a non reactive species to a reactive species (Bashan et al. 2009).

2.2.1.1 Generation of ROS

ROS are potentially toxic intermediates, which are usually increased under oxidative stress conditions. The highly reactive and more toxic type of ROS in the cell is OH•, which is produced from the interaction between O_2•- and H_2O_2. Haber-Weiss reaction is the major mechanism by which the OH• is formed (Kehrer 2000) as given in the following equation:

$$\dot{O_2}^- + H_2O_2 \rightarrow HO^\bullet + O_2 + HO^-$$

In addition to Haber-Weiss reaction, OH• can be produced via Fenton reaction in the most biological systems (Liochev 1999). Fenton reaction involves the use of the metal ion ($Fe^{2+/3+}$) catalyst to generate OH• in two steps as shown below.

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

While OH• is the most active type of ROS in the cell, O_2•- and H_2O_2 are the most common types of ROS that are produced during a series of physiological reactions in the cell. For instance, mitochondrial respiratory chain or the electron transport chain (ETC), cytochrome P450 family, xanthine oxidase, glucose oxidase, amino acid oxidase, fatty acid beta oxidation, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and oxygenase enzyme reactions, which use O_2 as a substrate, are known to generate huge amounts of ROS as a byproduct (Burton and Jauniaux 2011, Chandra et al. 2009, Fujii et al. 2005, Lu et al. 2012, Malhotra and Kaufman 2007, Schonfeld et al. 2010).

2.2.1.2 The origin of ROS in living cells

ROS can originate from several metabolic and enzymatic reactions in different cellular organelles such as mitochondria, peroxisomes, endoplasmic reticulum and plasma membrane (Figure 2) as follwing:

a. Mitochondria

Mitochondria are the power house of the cell. They are the main site of oxygen metabolism. The electron transport system of the mitochondria consumes about 85-90% of the total utilized oxygen by the cell, which are required for energy metabolism and involved in production of ATP via different pathways such as oxidative phosphorylation (OXPHOS) and fatty acid beta-oxidation. Therefore, high numbers of mitochondria are basically presented in all types of the cells (Shigenaga et al. 1994). On the other hand, they are the greatest source of oxidants and most of ROS (mainly: superoxide, hydroxyl radical and hydrogen peroxide) are produced by the mitochondria when electrons leak from the respiratory chain or ETC in the inner mitochondrial membrane (Cindrova-Davies et al. 2007, Fujii et al. 2005).

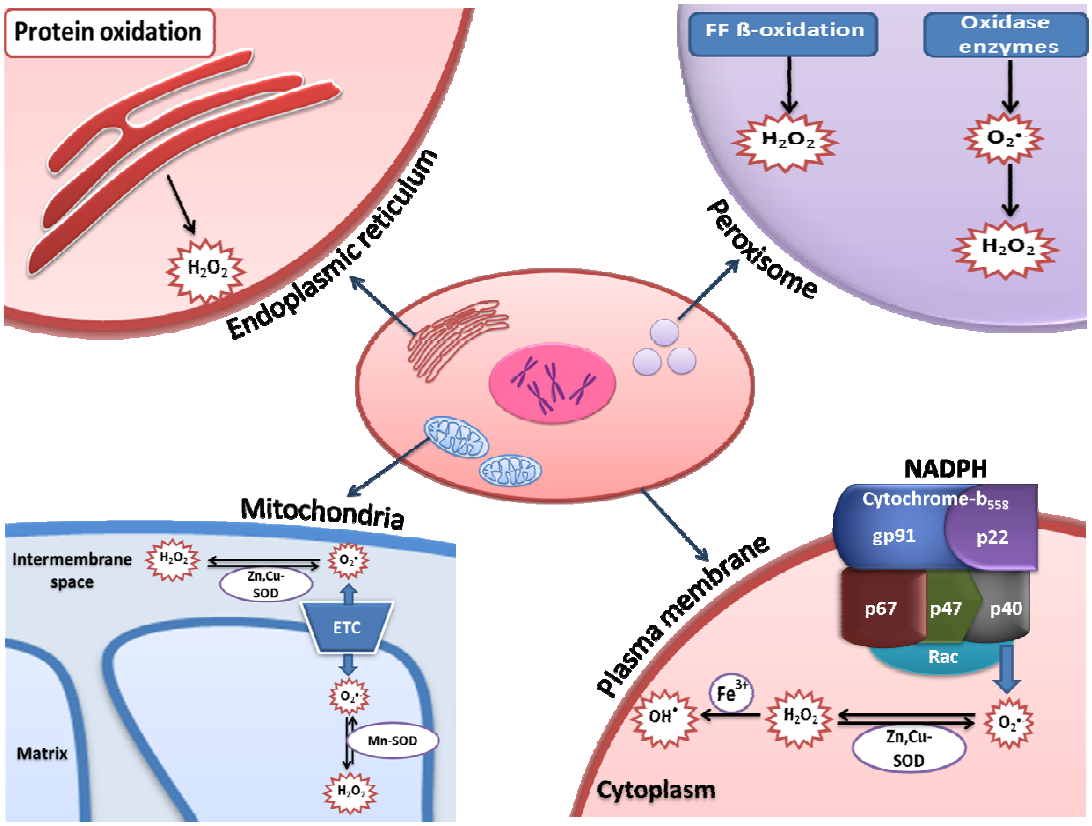


Figure 2: Schematic diagram showing different cellular sites of ROS generation including mitochondria, endoplasmic reticulum, peroxisome and plasma membrane.

The inner mitochondrial membrane expresses the rate of electron transport chain, which generates the membrane-impermeable superoxide anion. Mitochondrial ETC consists of four membrane-bound complexes called complex I (NADH-Q oxidoreductase), complex II (succinate-Q reductase), complex III (Q-cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase). These four membrane-bound complexes are linked by two small electron carriers namely ubiquinone (also called coenzyme Q) and cytochrome c (Berg et al. 2002). The membrane-bound complexes and other electron carriers, in the ETC, act as a molecular cable to transfer electrons from NADH and FADH to highly oxidizing dioxygen to form O_2•-. While complex IV dose not seem to be involved in ROS generation, superoxide anion can be produced via mitochondrial ETC by complexes I, II and III as shown in figure 3. Superoxide is generated into the mitochondrial matrix by complexes I and II, while complex III is able to generate superoxide into both the mitochondrial intermembrane space and the mitochondrial

matrix (Camello-Almaraz et al. 2006, Chandel and Budinger 2007, Droge 2002, Turrens 2003).

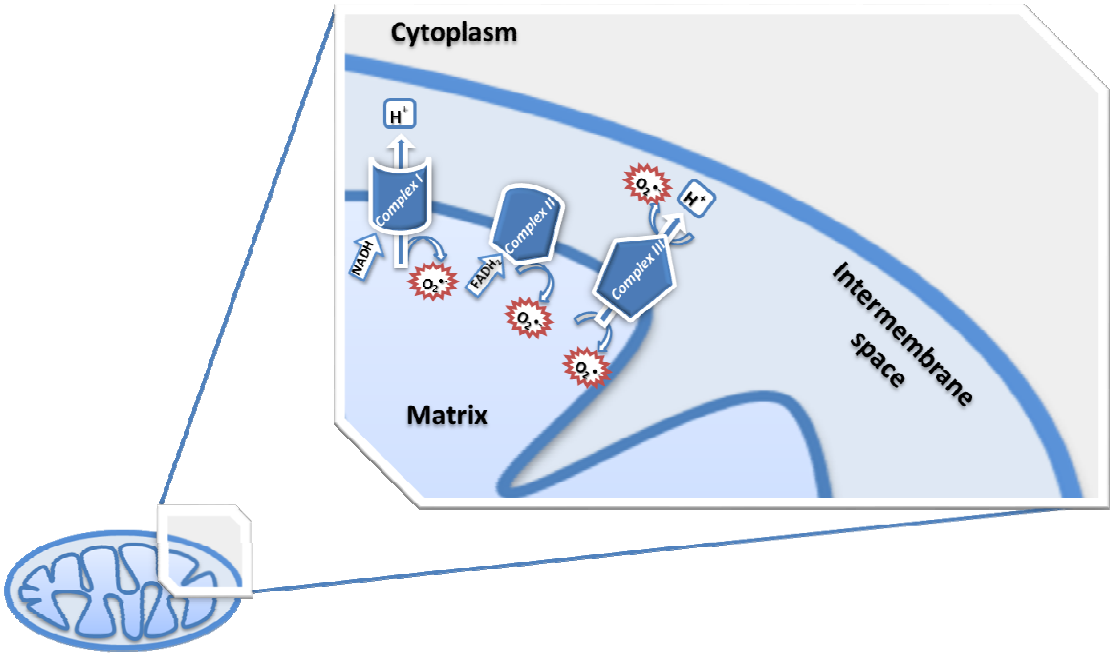


Figure 3: Generation of superoxide anion via mitochondrial electron transport chain by complexes I, II and III.

Superoxide is the precursor of most other reactive oxygen species. It can be then converted into H_2O_2 the via mitochondrial dismutation reactions (Figure 4). Superoxide dismutase enzymes, including Cu, Zn and Mn-SOD, can efficiently catalyze dismutation reaction in/outside the mitochondria to form hydrogen peroxide from the reaction of superoxide anion with water. Furthermore, H_2O_2 can also be diffused out of the mitochondria and into the cytoplasm. In the presence of iron in the cytoplasm, H_2O_2 can be converted to OH• through the Fenton reaction (Figure 4), as consequence, it can be able to attack the other cell organelles (Murphy 2009).

Mitochondrial dysfunction associated with the disturbed calcium homeostasis and enhanced cellular oxidative stress has long been recognized to play an essential role in cell damage (Frandsen and Schousboe 1993). Calcium is known to activate several cellular enzymes, many of which can stimulate the generation of endogenous ROS such as calcineurin, phospholipase A2, xanthine dehydrogenase, nitric oxide synthase and endonucleases. Furthermore, when calcium is taken up by the mitochondria, this can increase ATP production by stimulating the activity of matrix dehydrogenases.

However, an increase in ATP production and mitochondrial calcium levels can also enhance ROS generation (Kowaltowski et al. 1995, Rego and Oliveira 2003, Zoeteweij et al. 1992).

Although mitochondria have their own antioxidant mechanisms that are required for ROS scavenging, the generation rate of ROS inside mitochondria is higher than their antioxidant capability, which resulted in excessive ROS accumulation not only inside mitochondria but also in the cell cytoplasm (Kirkinezos and Moraes 2001, Melov et al. 1998, Nohl and Hegner 1978).

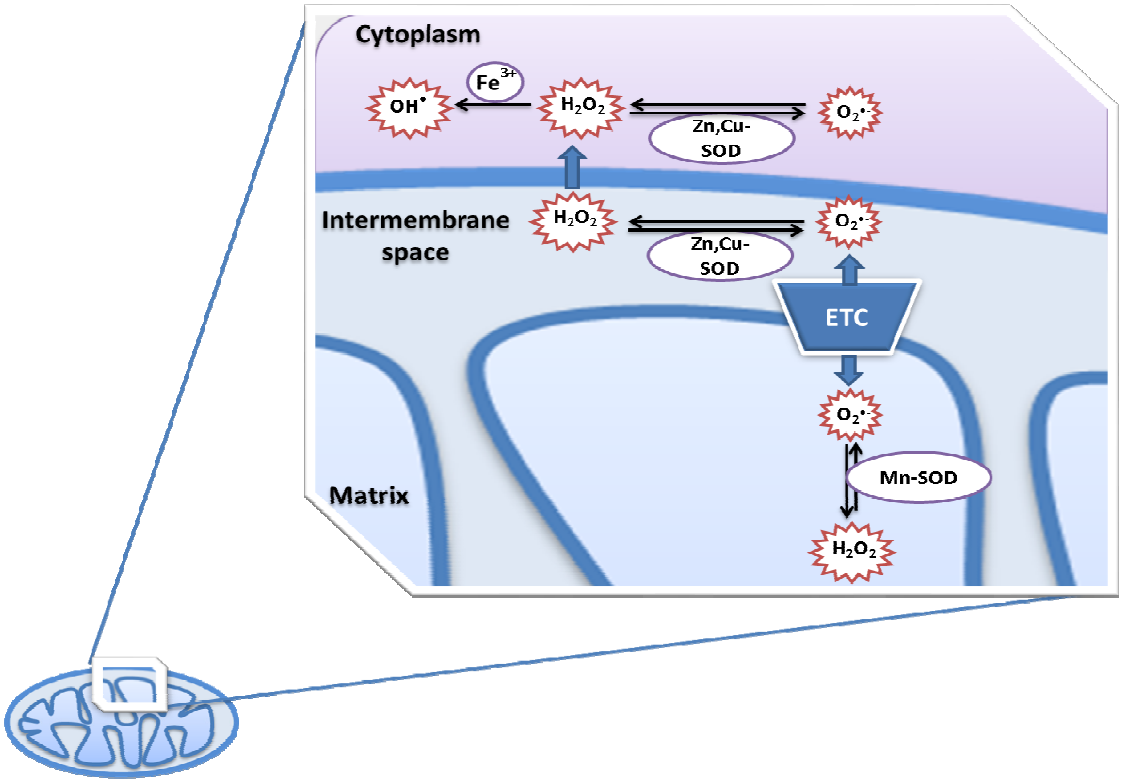


Figure 4: The conversion of superoxide anion to hydrogen peroxide and hydroxyl radical via dismutation reactions catalyzed by Cu, Zn and Mn-SOD enzymes in and outside the mitochondria.

b. Peroxisomes

In addition to mitochondria, the essential role of peroxisomes and their enzymes in ROS production has been well known ever since their discovery almost 40 years ago. Moreover, recent studies have revealed their involvement in the oxygen free and non

free radicals and nitric oxide metabolism (Schrader and Fahimi 2004, Singh 1996). The overall role of peroxisomes and their enzymes involved in ROS generation are briefly reviewed in table 1 (Schrader and Fahimi 2006).

Table 1: The peroxisomes enzymes involved in ROS generation

Enzyme	Substrate	ROS
(1) Acyl-CoA oxidases		
(a) Palimtoyl-CoA oxidase	Long chain fatty acids	H_2O_2
(b) Pristanoyl-CoA oxidase	Methyl branched chain fatty acids	H_2O_2
(c)Trihydroxycoprostanoyl-CoA oxidase	Bile acid intermediates	H_2O_2
(2) Urate oxidase	Uric acid	H_2O_2
(3) Xanthine oxidase	Xanthine	H_2O_2, $O_2\cdot^-$
(4) D-amino acid oxidase	D-Proline	H_2O_2
(5) Pipecolic acid oxidase	L-pipecolic acid	H_2O_2
(6) D-aspartate oxidase	D-aspartate, *N*-methyl-D-aspartate	H_2O_2
(7) Sarosine oxidase	Sarcosine, pipecolate	H_2O_2
(8) L-alpha-hydroxy acid oxidase	Glycolate, lactate	H_2O_2
(9) Poly amine oxidase	*N*-Acetyl spermine/spermidine	H_2O_2
(10) Nitric oxide synthase	L-Arginine	NO

c. Endoplasmic reticulum

Endoplasmic reticulum (ER) plays a vital role in several cellular activities. It is the central location of folding and assembly of the proteins, lipid biosynthesis and Ca^{2+} storage. In addition, ER has been reported as one of the main sites to generate ROS in the cells. Accumulating evidence suggests that generation of ROS as a byproduct of protein oxidation is one of the consequences of the protein folding process in ER. It has an exclusive oxidizing environment, as a result of the predominant disulfide bond formation during the protein folding process. It is well known that disulfide bond formation is driven by several proteins including ERO-1 (endoplasmic reticulum

membrane associated oxidoreductin-1), protein disulfide isomerase (PDI) and a novel conserved FAD-dependent enzyme. PDI, which is a member of the thioredoxin super family, catalyzes disulfide bond formation via series of reactions involving thiol-disulfide oxidation, reduction and isomerization (Frand and Kaiser 1999). Throughout this series of reactions, ERO-1 is oxidized by O_2 and acts as a specific oxidant of PDI. ERO-1 can recruit (FAD)-dependent reaction for transmitting electrons from PDI to O_2, which resulted in protein folding and leading to induce oxidative stress through the overproduction of ROS (Figure 5). This process is believed to generate about 25% of the total ROS production of the cell (Chaudhari et al. 2014, Malhotra and Kaufman 2007, Tu and Weissman 2004). Besides, ER stress-associated NADPH oxidases are also involved in the production of $O_2{\cdot}^-$ (Figure 5).

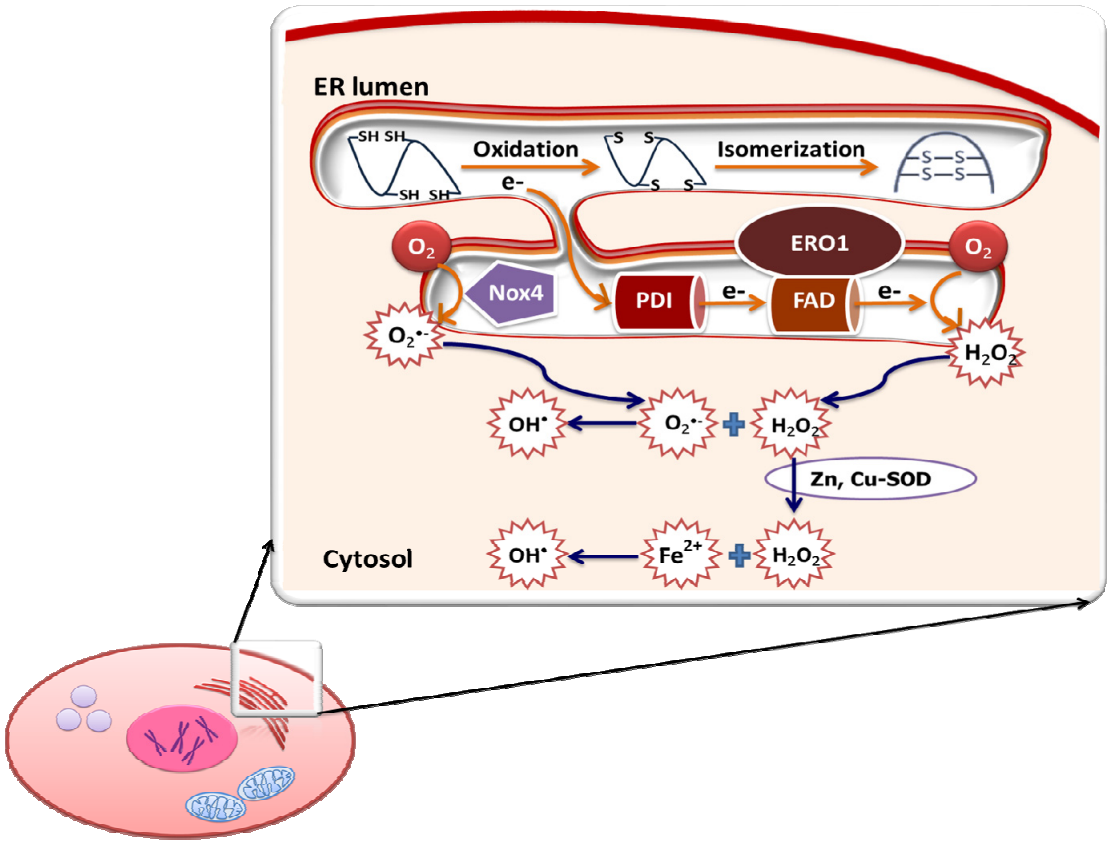


Figure 5: Generation of superoxide anion during the protein folding process and formation of hydrogen peroxide via NADPH oxidase 4 (Nox4) in endoplasmic reticulum (ER).

Although the role of ER stress-associated NADPH oxidases as an endogenous source of ROS is not yet fully explained, NADPH oxidase 4 (Nox4), which is one of the NADPH oxidase isoforms localized mainly in ER, has recently been considered as a possible source of ROS (Radermacher et al. 2013, Santos et al. 2009, Van Buul et al. 2005). In addition, the alterations in oxidative environment of the ER can enhance the ER stress, which result in an increased level of the generated ROS (Bhandary et al. 2013, Malhotra and Kaufman 2007, Ozgur et al. 2014, Santos et al. 2009, Yuzefovych et al. 2013).

d. Plasma membrane

Plasma membrane NADPH oxidase is one of the major sources of ROS in the cell. The NADPH oxidase is a protein complex formed by the assembly of resident membrane proteins ($gp91^{phox}$ and $p22^{phox}$ and cytochrom b588) and by the recruitment of cytoplasmic proteins including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small G protein Rac (Griendling et al. 2000). Recently, it has been evidenced that oxidative stress conditions stimulate plasma membrane NADPH oxidase to generate great amounts of O_2•- which can be subsequently converted to other types of ROS such as H_2O_2 (Chandel and Budinger 2007, Fan et al. 2007). Activation of the NADPH oxidase enzyme is associated with the assembly of the enzyme subunits at the membrane, where oxygen is reduced to superoxide (Bedard and Krause 2007, Finkel 2011) as in the following equation:

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP^+ + H^+$$

2.2.1.3 ROS activity

ROS types vary in their activity and their capability to react chemically with the other molecules. Basically, the more active ROS is the shorter its half-life, since it is faster to interact with other molecules. As a result, the distance which a specific type of ROS could move from its site of generation is different and associated with its activity. For example, H_2O_2 has a half-life of minutes and is considered a less reactive type of ROS. Thus, it can theoretically travel large distances and be able to cross the cells and tissues. On the other hand, the high reactivity of the short-lived OH• molecule decrease its diffusion distance to the range of a small protein (Bashan et al. 2009). In general,

hydrogen peroxide has half life in a range of minutes, while the peroxyl radical and nitric oxide have a half-life of seconds, peroxynitrite has a half-life of milliseconds, superoxide anion and singlet oxygen have a half-life of microseconds and the most active type of ROS is the hydroxyl radical with a half life of nanoseconds (Kehrer 2000).

2.2.2 Oxidative stress damage

The major consequence of increasing ROS production under oxidative stress conditions is the oxidative damage to DNA, proteins and lipids, which can strictly affect cell viability and eventually leading to cell death (Astley and Elliott 2005, Dalle-Donne et al. 2006, De Vizcaya-Ruiz et al. 2009, Kehrer 2000).

2.2.2.1 Oxidative damage to DNA

In any living cell, ROS can attack DNA and affect its structure and function, resulting in different types of DNA damage. For instance, ROS can induce double strand breakage, DNA structural alterations (i.e. deoxyribose modification), base pair mutations, insertions, deletions, rearrangements, sequence amplification (Cerutti et al. 1994, Cerutti 1994, Dizdaroglu 1993, Dizdaroglu et al. 1993, Epe et al. 1993) and DNA cross-linking (De Bont and van Larebeke 2004). In addition to nuclear DNA, oxidative damage could also be involved in the deletions and mutations in mitochondrial DNA. Even under normal conditions, oxidative damage to mitochondrial DNA (mtDNA) is extensive and its mutation rate is about 5 to 10 times of the rate seen in nuclear DNA (Richter et al. 1988). DNA of mitochondria encodes several proteins such as the enzymes involved in the electron transport chain. Therefore, high rate of mutations in mtDNA may lead to impaired energy production as well as mitochondrial dysfunction (Arnheim and Cortopassi 1992, Burton and Jauniaux 2011, Hegler et al. 1993).
The biochemical reactions that are likely to be involved in DNA damage are oxidation, methylation, depurination and deamination (Joenje 1987, Winterbourn 1995, Zuo et al. 1995). ROS and its reactive products such as hybrid reactive species can oxidize most of the DNA bases (Jena and Mishra 2005, Jena 2012, Kamiya 2003). For instance, peroxynitrite, which resulted from the chemical interaction between NO and O_2•-, can directly attack and damage to DNA bases by deamination and nitration of guanine

residues (Ohshima and Bartsch 1994, Routledge et al. 1994). For many years, methylation of DNA was believed to play a vital role in the regulation of gene expression during cell differentiation and embryonic development (Frostesjo et al. 1997, Heby 1995). Recent studies have generally provided clear evidence that transformation of guanine to 8- hydroxyguanine, as a result of ROS attack under stress conditions, has been found to change the enzymatic reactions that catalyze methylation of adjacent cytosines, as a consequence, providing an association between oxidative DNA damage and methylation pattern changes (Cooke et al. 2003, Dizdaroglu 1992, Dizdaroglu et al. 2002, Wiseman and Halliwell 1996).

2.2.2.2 Oxidative damage to protein

Protein and amino acids are a major target for ROS mediated oxidative stress damage because of their abundance and high reactivity with many species. ROS, especially O_2•-, H_2O_2, $^1O_2$, OH• and NO can interact with and attack proteins in both side chains of polypeptides and its backbone (Dalle-Donne et al. 2003, Davies 2005). Therefore, oxidative stress damage to protein comprises several chemical reactions such as amino acid side chains oxidation, polypeptide chains fragmentation, generation of cross linkages by the formation of dityrosine bridges. Most of these oxidative modifications are usually irreversible and can consequently have several harmful effects including major chemical and physical changes. Subsequently, these alterations can cause protein fragmentation, aggregation, abnormal folding, change the interactions with other molecules and eventually leading to loss protein functions (e.g., enzymatic, structural, or signaling) (Chao et al. 1997, Davies et al. 1995, Dean et al. 1986, Dean et al. 1997, Grant et al. 1993, Hawkins et al. 2002, Hawkins et al. 2003, Kuhn and Arthur 1999, Shen et al. 2000). Oxidative damage to proteins and amino acids, in some cases, is limited to specific residues, while with others (e.g., hydroxyl radicals) damage is prevalent, nonspecific and non-repairable (Davies 2000).

2.2.2.3 Oxidative damage to lipids

Lipids are attacked continuously by reactive species that can affect its structure and functions. ROS, mainly hydroxyl radicals, are able to oxidize fatty acids causing lipid peroxidation in all the cell organelles that contain lipids or polyunsaturated fatty acid side chains such as cell membranes. Recently, lipid peroxidation is considered as the most dangerous damaging process to cell structures in every living organism. This is mainly because of the nature of lipid peroxidation process which is a self-propagating chain-reaction and just the initial oxidation of a few fatty acid molecules can cause a series of dramatic damages to the cell (Gill and Tuteja 2010, Gutteridge 1995, Mylonas and Kouretas 1999). For instance, OH• reacts with hydrocarbons, such as the fatty acid side-chains of membrane lipids, to take out the hydrogen (H•) and leave behind a carbon-centred radical (C•) to start the free radical chain reaction as following (Aikens and Dix 1990, Cheeseman 1993, Dix and Aikens 1993):

$$\geq CH + OH\bullet \rightarrow \geq C\bullet + H_2O$$

The carbon-centred radicals (C•) react then with oxygen to generate peroxyl radical.

$$\geq C\bullet + O_2 \rightarrow \underset{\text{peroxyl radical}}{\geq C - O - O\bullet}$$

After that, peroxyl radical can attack other fatty acid side-chains in the cell membrane lipids.

$$\geq C - O - O\bullet + \geq CH \rightarrow \underset{\text{lipid hydroperoxide}}{\geq C - O - OH} + \geq C\bullet$$

The resultant carbon-centred radical (C•) reacts again with O_2 to generate another peroxyl radical and the chain reaction continues. Propagative lipid peroxidation is a well known example of oxidative breakdown to the biological lipids that occurs in most cellular membranes and organelles including plasma membrane, mitochondria, microsomes and peroxisomes. In addition, the end products of lipid peroxidation such as hydroperoxides can obstruct the protein synthesis, signal transduction, transport and change the enzymatic and metabolic activities (Dix and Aikens 1993, Fridovich and Porter 1981, Girotti 1998).

2.2.3 Oxidative stress and ROS generation during in vitro production of mammalian embryos

In the last decade, although a significant development achieved in in vitro maturation, fertilization and in vitro culture of mammalian embryos, the final success rates remain unchanged at 30%–40% blastocyst rate (Pasqualotto et al. 2004, Viuff et al. 1999). The main reason for this low success rate is the suboptimal environmental conditions under in vitro compared to the in vivo conditions. Several environmental conditions under in vitro culture system are supposed to be the source of oxidative stress for IVP embryos as manifested by the increased reactive oxygen species accumulation (Goto et al. 1993).

2.2.3.1 Endogenous source of ROS during IVP of mammalian embryos

Similar to the other types of cells, oxygen metabolism is important for early embryonic development. Gametes and embryonic cells utilize oxygen in the energy metabolism process, thereby, they are considered to be one of the major sources of ROS during in vitro production procedure (Balaban et al. 2005, Guerin et al. 2001).

Gametes

Low physiological levels of ROS may have a beneficial role during oocyte development and ovulation (Attaran et al. 2000, Oyawoye et al. 2003). However, speculation regarding the origin of the ROS during that process is still unclear. Moreover, the role of ROS during in vitro maturation of oocyte is still debated (Agarwal et al. 2006, Guerin et al. 2001, Gupta et al. 2009, Sugino et al. 2000). In contrast, evidence exists that the morphological abnormalities of the male gamete and leukocytes are major sources of ROS in human. Sperm intracellular mechanisms may generate ROS at the level of plasma membrane via NADPH oxidase system (Aitken et al. 1992). Furthermore, ROS can be produced in mitochondria via NADH-dependent oxidoreductase system (Gavella and Lipovac 1992) and cytochrome b5 reductase system activities (Agarwal et al. 2003, Gavella and Lipovac 1992). In addition, ROS can be extracellularly produced by leukocytes which are present in the seminal vesicles secretions (Aitken et al. 1995, Shekarriz et al. 1995b). The deleterious effects of ROS accumulation on sperm and

oocyte can be extended after fertilization to affect early embryonic development (Agarwal et al. 2006). It has been reported that during in vitro fertilization, oocyte incubation with a critical number of ROS-producing spermatozoa that remain outside the oocyte could cause oxidative damage to the oocyte and the resultant embryos (Alvarez et al. 1996).

Embryo

ROS can be generated in the embryo via different metabolic pathways and enzymatic systems activity such as mitochondrial oxidative phosphorylation pathway, NADPH oxidase and xanthine oxidase enzymes (Loutradis et al. 1987, Manes and Lai 1995, Nasresfahani and Johnson 1991, Thompson et al. 2000).

Oxidative phosphorylation

During the early embryonic development, pre-implantation embryos are developing very fast and consuming large amount of energy. And as in living aerobic cells, they generate the energy as adenosine triphosphate (ATP) molecules via mitochondrial OXPHOS and glycolysis processes. ETC of OXPHOS machinery comprises four protein complexes (I, II, III and IV). Three of these four complexes (I, II and III) are considered to be the main source of ROS produced in mitochondria (Huttemann et al. 2007). In the embryo, it has been reported that excessive ROS generation occurs as a by product of OXPHOS process at critical time points during the early embryo development, such as embryonic genome activation, embryonic compaction, blastocyst formation and embryo hatching, due to increased energy demands (Houghton et al. 1996, Morales et al. 1999, Nasresfahani et al. 1990b, Rozell et al. 1992, Thomas et al. 1997). For example, about 70% of the oxygen is metabolized at the blastocyst stage and less than 30% at time of the genome activation (2- to 4-cell stages) through mitochondrial OXPHOS in mouse embryos (Trimarchi et al. 2000). Moreover, it has been shown that inhibition of OXPHOS led to decrease ROS generation and has a positive impact on in vitro embryo development in both bovine and porcine embryos (Machaty et al. 2001, Rieger et al. 2002, Thompson et al. 2000).

NADPH oxidase

In addition to OXPHOS, oxidase enzymes activities (such as NADPH oxidase and xanthine oxidase) are present as sources of ROS during early embryo development. For example, NADPH oxidase has been shown to be involved in the production of O_2•- and H_2O_2 in rabbit blastocyst (Manes and Lai 1995), moreover, inhibition of NADPH oxidase in 2-cell mouse embryos induces a concentration-dependent reduction in H_2O_2 production (Nasresfahani and Johnson 1991). In bovine, supplementation of the culture medium with vitamin E, which is an antioxidant and can protect cell against oxidative damage, improved embryo development and blastocyst formation rate by 6%. This improvement could be due to the inhibition of NADPH oxidase (Olson and Seidel 2000a).

Xanthine oxidase

Xanthine oxidase is another important enzyme that has a vital role in the generation of ROS in the cell (Sato et al. 2011). This enzyme is involved in adenosine metabolism, converting hypoxanthine to xanthine and xanthine to uric acid, with the concomitant generation of superoxide and hydrogen peroxide (Many et al. 1996). Although the contribution of xanthine oxidase enzyme in ROS production is relatively low compared with that of the NADPH oxidases under normal physiological conditions (Bevilacqua et al. 2012), the presence of hypoxanthine/xanthine oxidase in the in vitro culture medium dramatically inhibit the early embryo development, decrease cleavage rates and causes a 2-cell block in mouse embryos (Fukuhara et al. 2008, Loutradis et al. 1987). Thus, cellular oxidative damage by the xanthine oxidase-mediated generation of ROS might explain the incidence of embryonic arrest during the in vitro production of the mammalian embryos (Agarwal et al. 2006, Aiken et al. 2008, Johnson and Nasr-Esfahani 1994, Nasresfahani and Johnson 1991). In addition, the inhibition of this enzyme induces a decrease in ROS production in mouse embryos. Therefore, xanthine oxidase has been suggested to be the main source of ROS during early embryonic development of in vitro produced mouse embryos (Nasresfahani and Johnson 1991). In bovine embryos, ROS generation is caused, in part, by the presence of high concentration of glucose in the embryo culture medium (Iwata et al. 1998). Previously,

glucose has been shown to inhibit the hypoxanthine phosphoribosyl transferase (HPRT) activity and induce developmental arrest in mouse embryos. Inhibition of HPRT activity, which is involved in salvaging purine and decreasing of ROS levels, induces the formation of xanthine from hypoxanthine via xanthine oxidase enzyme and increase the generation of superoxide anions (Downs and Dow 1991).

2.2.3.2 External factors inducing ROS generation during IVP of mammalian embryos

Several external factors contribute to the induction of oxidative stress and increasing the generation of ROS during IVP of mammalian embryos (Figure 6). In addition to in vitro culture atmosphere that are surrounding the oocyte and embryos (e.g. oxygen concentration in the culture system atmosphere, visible light and pollutants), culture medium components and supplements and the nature of in vitro procedure itself can also enhance the ROS production in IVP embryos (Agarwal et al. 2006).

In vitro culture atmosphere inducing ROS generation

Oxygen concentration

Oxygen is necessary for aerobic respiration to generate ATP, which is required for all cell functions. However, changes in oxygen levels are known to induce oxidative injury and affect the life span of the cell (Vonzglinicki et al. 1995). Therefore, in vitro culture atmosphere in which an embryo is grown during the in vitro production of the mammalian embryos is a critical factor affecting the outcome of the culture system. Oxygen concentration used in the in vitro culture system atmosphere was cited as the most potential and important external factor that can enhance ROS generation. It may affect gametes and embryo metabolism as well as embryo quality, viability and developmental competence (Booth et al. 2005, Dumoulin et al. 1999, Kitagawa et al. 2004, Leoni et al. 2007, Liu and Foote 1995, Orsi and Leese 2001, Thompson et al. 1990). Under in vivo conditions in the oviduct, oxygen concentration is around 25 to 30% of the atmospheric oxygen tension (Fischer and Bavister 1993). Two established gas systems are widely used in the incubators during in vitro embryo production process. One is under low (5%) oxygen tension as in oviduct and the other is under the

atmospheric oxygen concentration (20%). Under in vitro culture system, it is too expensive to control oxygen concentration to 5%. That is why in most IVP laboratories 20% oxygen level is successfully used to produce IVP embryos especially in cattle (Karja et al. 2004, Suzuki et al. 1999, Varisanga et al. 2002). On the other hand, IVP of embryos under high oxygen concentration (20%) promotes oxidative stress to the embryo and leading to a rise in ROS level compared to 5% O_2 culture system (Dalvit et al. 2005). Several studies have elucidated the effect of oxygen level in the culture system during the in vitro production of embryos 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 + 5% CO_2 + 90% N_2) than those that were generated under higher oxygen concentration (air + 5% CO_2) in bovine (Liu and Foote 1995, Thompson et al. 1990), porcine (Booth et al. 2005), mouse (Orsi and Leese 2001) and human (Dumoulin et al. 1999). Similarly, high oxygen concentration (20 vs 5% O_2) during IVF was found to be unfavorable for the production of high quality ovine blastocysts (Leoni et al. 2007). Atmospheric oxygen is freely dissolved in the in vitro culture medium (Newby et al. 2005). Therefore, O_2 concentration in the culture medium equilibrated with atmospheric oxygen is approximately 200 μM, which is considerably higher than the normal O_2 concentration (28-42 μM) within the cells (Oller et al. 1989). Many oxidases enzyme reactions that produce ROS in the cells are known to be oxygen dependent. Thus, high O_2 concentration in the culture media atmosphere may enhance some enzymatic reactions, such as xanthine oxidase and NADPH oxidase activity, resulting in an increase in the ROS levels within embryonic cells (Goto et al. 1993, Lopes et al. 2010).

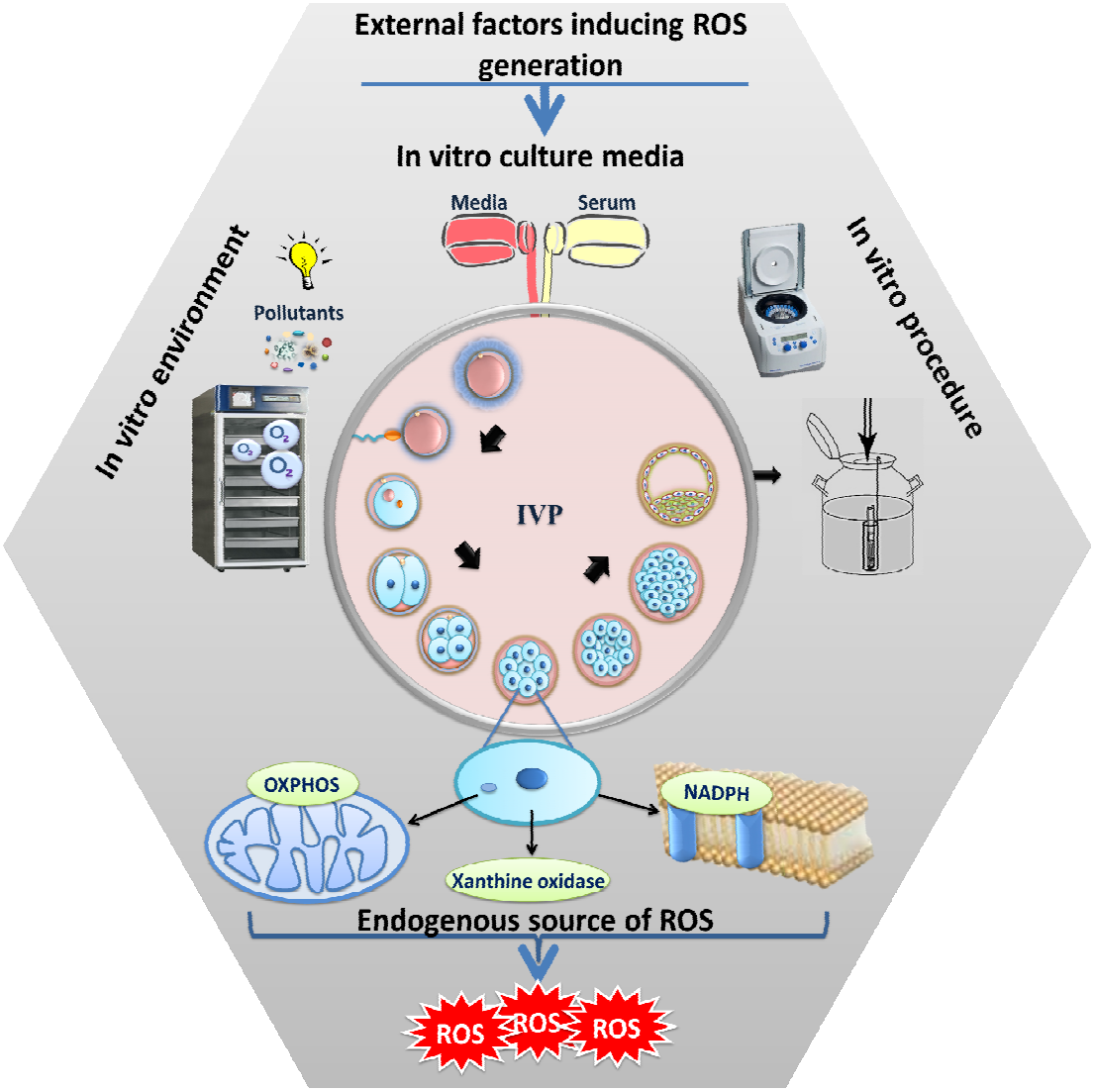


Figure 6: External factors influence the oxidative stress generation and enhance the generation of ROS during the in vitro production of mammalian embryos.

Visible light

Visible light is another external factor in the culture environment that may induce ROS production during in vitro production of the mammalian embryos (Goto et al. 1993). It was hypothesized that visible light can induce photodynamic stress and leading to oxidative damage to unsaturated lipids and sterols within the membranes (Girotti 2001) and oxidation of bases as well as breakdown of the DNA strands (Beehler et al. 1992).

In support of this hypothesis, an exposure of mouse embryos to visible light was dramatically sufficient to increase H_2O_2 level (Goto et al. 1993).

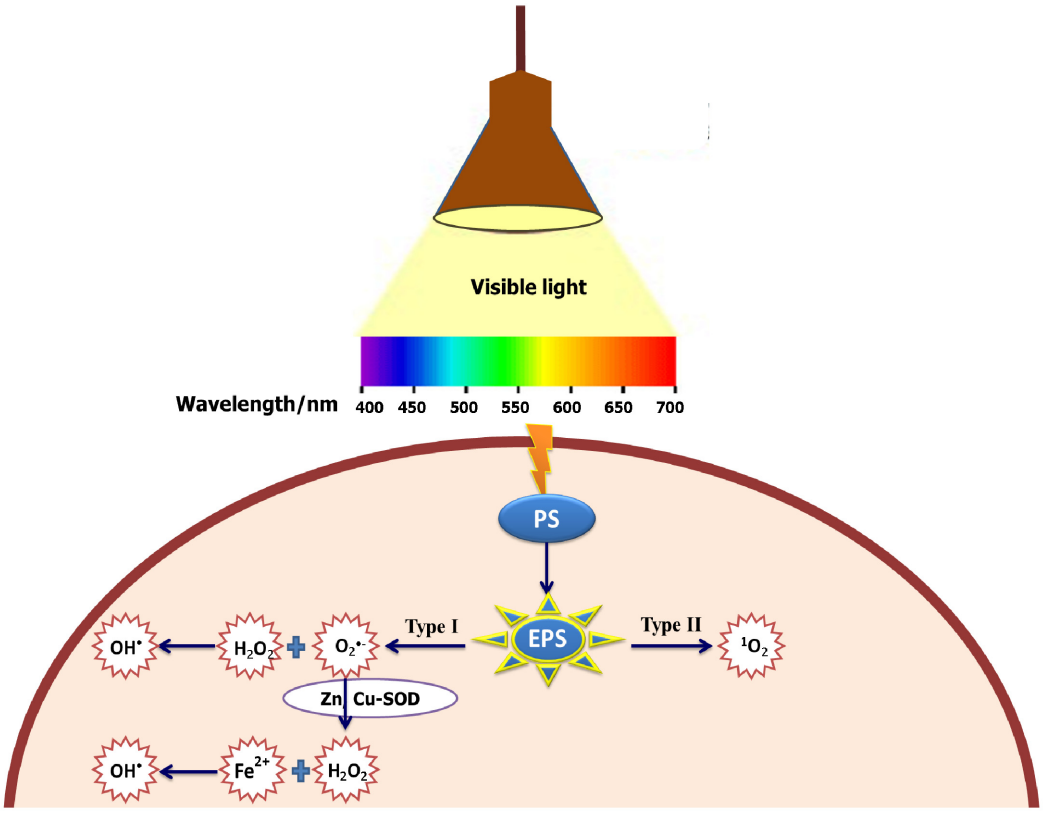


Figure 7: Mechanisms leading to ROS generation induced by visible light. Visible light (400–700 nm) exposure can induce ROS production via two types of photooxygenation reactions. The endogenous photosensitizers (PS) are light-sensitive molecules that enter an excited state after exposure to visible light to be excited photosensitizers (EPS). EPS participate in electron transfer processes, resulting in formation of O_2•- via type I reactions, or react with molecular oxygen, starting cascade of energy transfer processes that ultimately result in generation of $^1O_2$ through type II reactions.

Light-induced ROS generation mechanisms in embryos are still unclear. In mammalian cells, visible light exposure (400–700 nm) was reported to induce the production of ROS as a result of several photooxygenation reactions (Hockberger et al. 1999, Liebel et al. 2012). The key players in the photooxygenation reactions are the endogenous photosensitizers, such as porphyrins and flavins (Ravanat et al. 2000), which are a light-

sensitive molecules that enters an excited state after exposure to light of a specific wavelength. The excited sensitizers can then react with molecular oxygen, starting either a cascade of energy transfer processes that ultimately result in generation of non-radical but highly reactive $^1O_2$ (Type II reactions), or participating in electron transfer processes to biological substrates (such as membrane lipids) and solvent molecules or oxygen, leading to radicals and radical ions formation such as O_2•- (Type I reactions). These radicals can interact with other reactive molecules to produce hydroxyl and hydrogen peroxide radicals, as shown in Figure 7 (Buytaert et al. 2007, Foote 1991, Henderson and Dougherty 1992). Type I and II reactions can lead to oxidative damage of several cellular components and finally to cell death (Buytaert et al. 2007, Moor 2000).

Environmental pollutants

Many sources of ROS exist in the IVP of embryo laboratories, which induce ROS generation. Xenobiotics are foreign chemical compounds that have found their way into culture environment as environmental pollutants. They can induce different biological effects, including oxidative stress, toxicity, immunologic reactions and pharmacologic responses. Xenobiotics induced oxidative stress may be present in the air and/or water of the incubators used in IVP of the embryos (Agarwal et al. 2006, Cohen et al. 1997, Wells et al. 2009). It has been proved that air quality is important for success IVP systems. Moreover, unfiltered external air seems to be cleaner than laboratory air, which is the main source of the ambient air in the incubators (Cohen et al. 1997). This may be due to the accumulation of volatile organic compounds (VOCs) derived from some laboratory equipment (e.g. sterile petri dishes, compressed CO_2), other supplies, materials and devices, such as anaesthetic gases, refrigerants, cleaning agents, hydrocarbons and aromatic compounds (e.g. benzene and toluene) which can be destructive to embryo development in vitro.

The filtration of atmospheric air by intra-incubator air purification system significantly increased pregnancy rate following transfer of in vitro-produced bovine embryos to recipients (Khoudja et al. 2013, Merton et al. 2007). Therefore, IVP laboratories should be well equipped with high efficiency particulate air (HEPA) and efficient filters plus positive pressure for air particulate control (Khoudja et al. 2013). In addition, the low

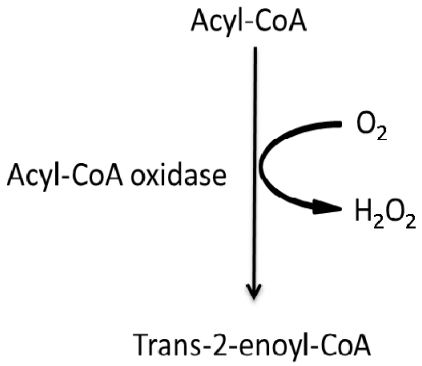
concentrations of some of these pollutants in incubator air may suggest the potential absorption of these pollutants by the water or culture medium (Cohen et al. 1997).

Culture media components inducing ROS generation

Culture media components used during IVP of mammalian embryos can also contribute to ROS production. Commercial culture media can generate ROS subject to their composition, which can directly affect the oocyte and embryo quality (Agarwal et al. 2006). Some of them may contain Fe^{2+} and Cu^{2+} metallic ions which are participating in the Fenton and Haber–Weiss reactions and can accelerate the generation of ROS within the cells. Moreover, Fe^{2+} can also attack lipids directly and enhance lipid peroxidation reactions leading to induction of peroxidative damages in lipid membrane structures once this has been initiated by free hydroxyl radicals. This may cause an in vitro block to development of the pre-implantation embryo (Nasresfahani et al. 1990a). Culture media supplementation with metal chelators such as transferrin and ethylenediamine tetra-acetic acid (EDTA) could be a possible way to decrease the detrimental effects of these ions (Nasresfahani et al. 1992, Orsi and Leese 2001).

Several supplements are commonly added to the culture medium for different reasons to improve the outcome of the IVP of mammalian embryos. Serum is one of the most important culture media additives due to its composition (in terms of lipids, proteins, amino acids, growth factors, etc.). Several investigators have shown that culture media supplementation with serum can accelerate embryo development and resulting in early blastocyst formation in culture (Carolan et al. 1996, Enright et al. 2000, Rizos et al. 2002b, Viuff et al. 1999). Furthermore, in long-term, serum has an effect on fetal development in cattle and sheep (Behboodi et al. 1995, Farin et al. 2001, Young et al. 1998). In contrary, others have observed a dual effect "biphasic effect" of serum during the development of the in vitro cultured bovine and ovine embryos. For instance, addition of serum in culture media can inhibit the early cleavage divisions (first and second cleavages through the 4-cell stage) and stimulate the embryo development after the embryonic genome activation "8- to 16-cell stages in bovine" (Rizos et al. 2003, Rooke et al. 2007, Thompson et al. 1998). The deletarious effect of serum may be attributable to the high levels of amine oxidase enzyme, which is present in serum and can induce ROS production (Agarwal et al. 2006, Parchment et al. 1990, Shannon

1978). Another major component of serum is the free fatty acids. Earlier studies showed that fatty acid composion of embryos derived from serum supplemented media is similar to fatty acid composion of the added serum. It has been suggested that serum to be the main sourec of fatty acids in serum-exposed embryos and the embryonic cells in culture readily take up fatty acids from serum-containing media (Sata et al. 1999, Spector et al. 1972, Spector and Soboroff 1972). The absorbed fatty acids are oxidized to generate energy via fatty acid beta-oxidation process which occurs in both mitochondria and peroxisomes. In the initial step of fatty acid beta-oxidation, an acyl-CoA is converted to trans-2-enoyl-CoA. This reaction is catalyzed by acyl-CoA oxidase enzyme and the electrons removed by oxidation pass to oxygen directly to produce H_2O_2 (Rao and Reddy 2001, Rosenthal and Glew 2009) as shown below:



In vitro procedure inducing ROS generation

A potential source of ROS during the in vitro production of mammalian embryos procedure is its generation during the preparations. For instance, spermatozoa are normally centrifuged during in vitro fertilization preparations. Centrifugation has been demonstrated to increase ROS generation in male gametes (Shekarriz et al. 1995a). Moreover, spermatozoa used for in vitro fertilization are likely to be derived from an environment experiencing oxidative stress. Some of these sperm may already have oxidative damage before semen preparations (Fingerova et al. 2009, Iwasaki and Gagnon 1992, Lopes et al. 1998, Pasqualotto et al. 2004, Saleh et al. 2003). Incubation of oocytes during in vitro insemination with a critical number of those spermatozoa that remain outside the oocyte could also induce oxidative damage to the oocytes and embryos (Alvarez et al. 1996).

Cryopreservation of gametes and embryos inducing ROS generation

Cryopreservation of embryo and gametes with limited viability loss is necessary to produce and transfer the in vitro mammalian embryos successfully. A recent study showed that frozen/thawed embryos exhibited significantly reduced pregnancy rate and calving results compared with the fresh counterparts (Agca et al. 1998). Moreover, cryopreservation of sperm triggered the antioxidant defenses loss and increased the DNA fragmentation compared to the fresh testicular sperm (Bilodeau et al. 2000, Dalzell et al. 2004). In oocytes, cryopreservation resulted in developmental arrest for the resultant embryos (Vanblerkom and Davis 1994). The freeze-thaw process during cryopreservation procedure is a severe stressor that can increase DNA damage and fragmentation levels (Thomson et al. 2009), modify the structure (e.g. plasma membrane) and integrity of the cell (Hendriks et al. 2014, McCarthy et al. 2010). One of the traumas by which the cryopreservation can affect the gametes and embryo competence is through the induction of oxidative stress by increasing ROS generation (Alvarez and Storey 1992, Lane et al. 2002, Rahimi et al. 2003, Zhao et al. 2012). Supporting that, ROS scavengers supplementation have been reported to protect gametes and embryos from the detrimental effects of the freeze-thaw process (Lane et al. 2002, Moubasher et al. 2013). For instance, supplementation of cryopreservation or preparation medium of spermatozoa with antioxidants, such as quercetin (Zribi et al. 2012), catalase (Moubasher et al. 2013), vitamin E (Taylor et al. 2009) and biotin (Kalthur et al. 2012), seemed to protect spermatozoa from oxidative stress damage during the freeze-thaw process, which resulted in significant improvement in spermatozoa motility, viability and DNA integrity and ultimately increased sperm survival. In addition, cryopreservation solutions supplemented with antioxidant namely ascorbate resulted in reduced ROS levels and significantly ($P<0.05$) increased blastocyst re-expansion and attachment (87.9% and 78.9% respectively) compared to the control group of embryos frozen without ascorbate (63.9% and 52.8% respectively) (Lane et al. 2002).

2.2.4 Deleterious effects of oxidative stress during IVP of mammalian embryos

Excessive ROS accumulation under in vitro culture conditions can overpower the cell natural antioxidant defenses and create an environment inappropriate for successful IVM, IVF and IVC. This is due to the presence of additional external factors that can increase the generation of ROS under in vitro conditions compared to in vivo conditions (Bedaiwy et al. 2004, Du and Wales 1993, Guerin et al. 2001, Johnson and Nasr-Esfahani 1994, Khurana and Niemann 2000, Liu and Keefe 2000). Multiple deleterious effects have been observed for exposing gametes and embryos to oxidative stress during all the steps of the in vitro production process and subsequent pregnancy outcome after embryo transfer, as shown in Figure 8 (Agarwal et al. 2005a, Ali et al. 1993, Ali et al. 2003, Alvarez et al. 1996, Bedaiwy et al. 2004, Cetica et al. 2001, Li et al. 1993, Liu and Keefe 2000, Luvoni et al. 1996, Nasresfahani et al. 1990b, Nasresfahani and Johnson 1991).

2.2.4.1 Effect of oxidative stress on oocyte and sperm

High ROS levels under oxidative stress conditions have been suggested to enhance chromosomal errors and induce the meiotic arrest of the oocytes (Downs and Mastropolo 1994, Tarin et al. 1996). Incrementally, sufficient evidence has accumulated to show that ROS can deteriorate oocyte quality and competence (Goud et al. 2008, Rajani et al. 2012, Tamura et al. 2008, Tamura et al. 2012). For example, incubation of mouse oocytes with 100 μM HOCl led to loss their viability as seen from dark cytoplasm and obvious signs of membrane damage (Goud et al. 2008). In addition, a direct effect of H_2O_2 on oocyte maturation has recently been reported (Tamura et al. 2008). In that study, oocytes from mice were cultured in vitro in maturation medium treated with different H_2O_2 concentrations. The percentage of mature oocytes with a first polar body was significantly decreased by incubation with H_2O_2 in concentration of 300 μM for 12 h. On the other hand, this inhibitory effect of H_2O_2 on oocyte maturation was blocked when oocytes were incubated with antioxidant namely melatonin in concentration of 10 ng/ml. An in vivo rat model treated with Neem leaf extract (NLE) exhibited oocyte morphological apoptotic changes including DNA and cytoplasmic fragmentation. The reasons for that was the increase of H_2O_2 levels which activates p53

and induces the expression of pro-apoptotic Bcl2-associated X protein (Bax) that modifies mitochondrial membrane potential and enhances cytochrome c release. The increased level of cytoplasmic cytochrome c leads to induces caspase-9 and caspase-3 activities that trigger destruction of specific proteins leading to DNA and cytoplasmic fragmentation and thereby oocyte apoptosis (Tripathi et al. 2012). The same oocyte morphological apoptotic changes were observed in in vitro rat model when oocytes were incubated with NLE (2.5 to 20.0 mg/ml) for 3.0 h (Chaube et al. 2006). On the other hand, oocyte incubation with maturation medium supplemented with cysteamine (100 μM), cysteine (0.6 mM), or beta-mercaptoethanol (β-ME) in a concentration of 100 μM in bovine (de Matos et al. 1996, Dematos et al. 1995) and glutathione reduced ethyl ester (GSH-OEt) in a concentration of 3 and 5 mM in bovine and macaque (Curnow et al. 2010, Curnow et al. 2011) increased the oocyte antioxidant capacity due to its stimulatory effect on GSH synthesis. The stimulation of GSH in the oocyte during IVM protected against oxidative stress in later stages of fertilization, improved embryo development and quality (Curnow et al. 2010, Curnow et al. 2011, de Matos et al. 1996, Dematos et al. 1995). This led to produce more early bovine embryos reaching the blastocyst stage, which are more resistant to oxidative damage induced by cryopreservation and most suitable for freezing. As a consequence, the embryo viability, in terms of re-expansion rates of blastocysts after 24-48 h of culture after cryopreservation, was significantly increased from 76 to 95 % (de Matos et al. 1996). In macaque, oocyte maturation in medium supplemented with GSH-OEt (3 and 5 mM) supported higher fertilization rates and blastocyst development and quality, in terms of increased blastocyst total cell and ICM cell number, in early maturing oocytes (4-6 h of culture oocytes) compared to late maturing counterparts (18-20 h) (Curnow et al. 2011).

ROS generated either internally, during normal metabolic activity in different cell organelles, or under the induction of several external factors, which can induce oxidative stress, can cause oxidative damage that impairs the mitochondria capability to perform their metabolic functions (Balaban et al. 2005, Ballinger 2005, Sahin and DePinho 2010). Mitochondrial metabolic capacity is believed to be one of the major factors that control the oocyte quality and developmental competence (Ge et al. 2012, Wang et al. 2007, Wang et al. 2009). During in vitro maturation, inhibition of mitochondrial metabolic capacity of immature mouse oocytes resulted in a decreased number of oocytes with nuclear maturation, reduced normal spindle formation and

chromosome alignment and subsequent poor oocyte quality (Ge et al. 2012). In addition, inhibition of mitochondrial functions in pig oocytes led to reduced ATP production, as a result of the low mitochondrial membrane potential, decreased extrusion of the first polar body, affecting the ability of immature oocytes to reach metaphase II (Lee et al. 2014). Not only that, but the effect of inhibited mitochondrial functions in oocyte is also controversial and can influence the embryo quality and developmental competence of the later developmental stages. For instance, during in vitro production of both mouse and pig embryos, loss of mitochondrial functions resulted in a significant reduction in the blastocyst formation rates (Ge et al. 2012, Lee et al. 2014).

In addition to female gametes, poor fertilization rates were found to be associated with elevated ROS accumulation in male gametes, which also resulted in further impaired embryo development and reduced pregnancy rates (Zorn et al. 2003). This reduction in the fertilization rates maybe due to the loss of plasma membrane fluidity as well as sperm penetration ability. Moreover, high ROS level can also induce oxidative damage to sperm DNA and impair the mitochondrial membrane potential which are known to be initial events in the cell apoptosis cascade (Agarwal et al. 2005a, Baker and Aitken 2005, Duru et al. 2000, Kim et al. 2013, Sharma et al. 2004, Twigg et al. 1998).

2.2.4.2 Effects of oxidative stress on IVP embryos

High oxidative stress under in vitro culture conditions can negatively affect early embryonic development in different ways. ROS are able to diffuse through cell membranes and can attack the most vital cellular molecules such as DNA, protein and lipids. This can also influence many organelles dysfunction including mitochondria and plasma membrane, which interfere with several metabolic pathways and lead to defective embryonic development (Bedaiwy et al. 2004, Du and Wales 1993, Johnson and Nasr-Esfahani 1994, Khurana and Wales 1989, Khurana and Niemann 2000, Liu and Keefe 2000).

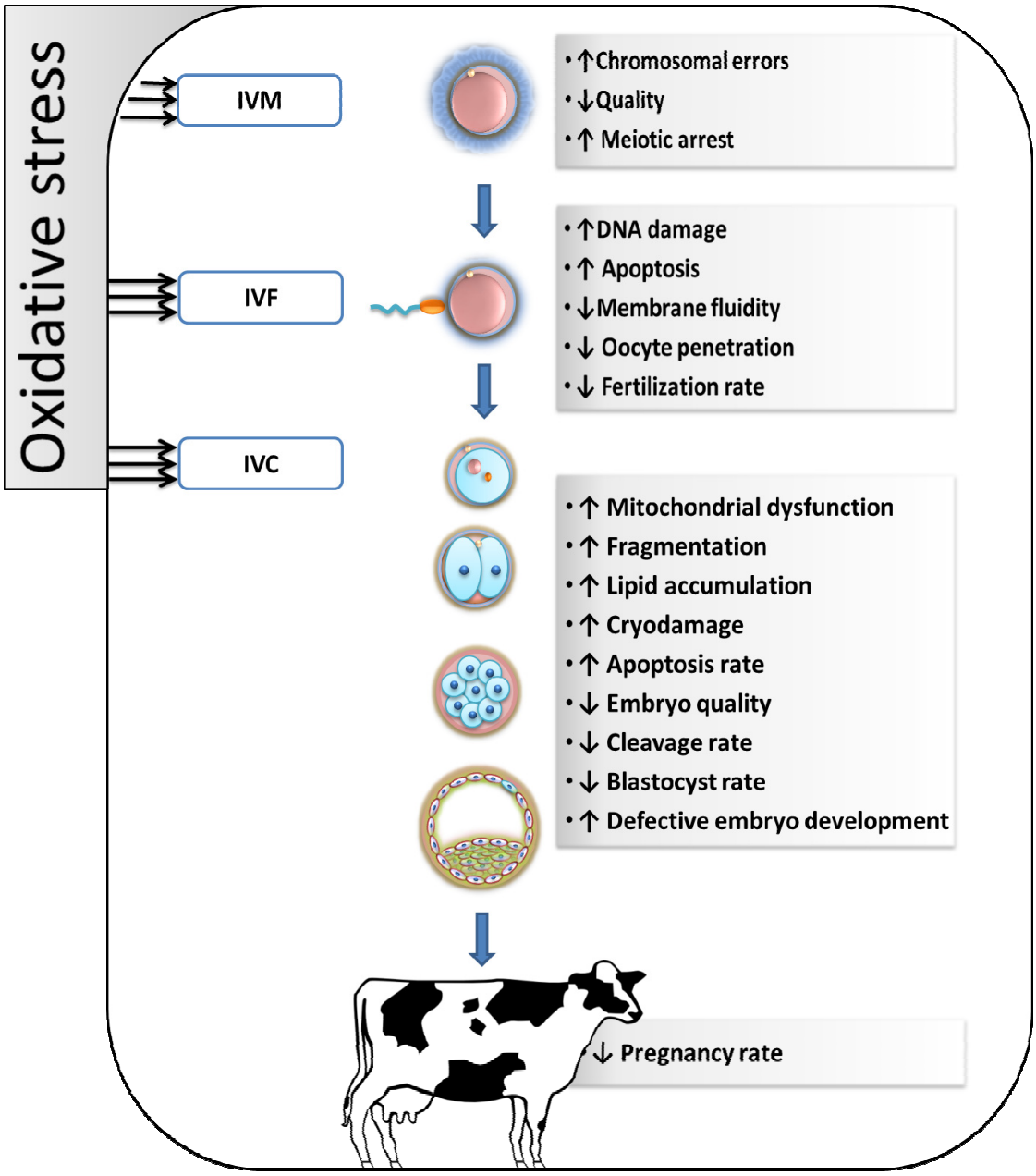


Figure 8: Detrimental effects of oxidative stress during IVM, IVF and IVC of IVP embryos and subsequent pregnancy outcome.

Mitochondrial dysfunction

Following fertilization and during early stages of embryonic development, the embryo is dependent on the function of efficient mitochondria (Chappel 2013, Ge et al. 2012). In addition to being a major site of energy metabolism to generate ATP, mitochondria

may also be a regulatory factor in other processes involved in the establishment of oocytes and early embryonic development, including modulating Ca^{2+} signaling and apoptosis (El Shourbagy et al. 2006, Jurisicova and Acton 2004, Mitchell et al. 2009b, Thompson et al. 1996, Thompson et al. 2000, Van Blerkom et al. 2006, Wilding et al. 2001). Optimal metabolism in mitochondria contributes, in part, to oocyte and embryo quality and subsequent development (Barnett and Bavister 1996, Leese et al. 2008, Leese 2012). ROS generated either by mitochondria or other cellular sources can cause oxidative damage that impairs the mitochondria ability to carry out their metabolic functions (Balaban et al. 2005, Ballinger 2005, Sahin and DePinho 2010). Oxidative damage to mitochondrial proteins, lipids and mtDNA result in mitochondrial dysfunction. Several mitochondrial enzymes or enzyme complexes which are sensitive to stress conditions can be inhibited by ROS such as aconitase, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase and membrane-bound complex I, II and III. Most of these proteins contain important sulfhydryl groups which are essential for their enzymatic activity and are subject to possible oxidation (Zeevalk et al. 2005). In addition, oxidative damage can inactivate mitochondrial DNA polymerase γ and slow mtDNA replication. It has been reported that one hour treatment with 250 μM H_2O_2 resulted in critical modifications in amino acid residues of DNA polymerase γ and significantly inhibited DNA polymerase activity as well as mitochondrial DNA replication in human fibroblast cells (Graziewicz et al. 2002). Inhibition of mtDNA replication results in a decline in their functions, which in turn leads to overproduction of ROS in mitochondria and further damage to mtDNA (Cui et al. 2012, Shokolenko et al. 2009) and accelerate energy depletion as well as cell death (Fleming et al. 1982, Miquel et al. 1980). Moreover, oxidative damage results in higher mtDNA mutation and deletion rates (about 10 fold) compared to nuclear DNA. This may be due to the absence of its protective histones that normally quench ROS and the lack of repair enzymes for mtDNA, which may explain its sensitivity to oxidative stress (Lee and Wei 2005, Richter et al. 1988, Taanman 1999). As mtDNA encodes indispensable components involved in OXPHOS and protein synthesis machinery (Falkenberg et al. 2007), accumulation of mtDNA mutations can disturb all mitochondrial functions and limit energy production in the cell. As consequence, the cell has a reduced capacity to support the other biological processes, leading to arrest normal cell division and

possibly apoptosis (Fleming et al. 1982, Hiona and Leeuwenburgh 2008, Miquel et al. 1980, Sastre et al. 2000).

Mitochondrial efficiency to generate ATP as a source of energy is required for different cellular processes in developing embryos, including DNA replication, cell division and genome activation (Wilding et al. 2001). Therefor, oocyte and embryo developmental competence in human (Wilding et al. 2001), bovine (Tarazona et al. 2006), porcine (El Shourbagy et al. 2006) and mouse (Ge et al. 2012, Mitchell et al. 2009a, Mitchell et al. 2009b, Wakefield et al. 2011) is dependent on the efficiency of the mitochondria. Moreover, loss of mitochondrial efficiency led to impaired glycolysis, tricarboxylic acid cycle (TCA) activity, reduced respiration rate, increased ADP levels ($P < 0.05$), decreased ATP production and ATP:ADP ratio (Wakefield et al. 2011). Low energy production is associated with not only the increased number of oocytes showing abnormal spindle formation and chromosome alignment as well as poor oocyte quality, but also a reduction in the number of embryos that reached the blastocyst stage, the blastocyst cell number ($P < 0.01$) and the numbers of both TE and ICM cells ($P < 0.05$ and $P < 0.01$, respectively) (Ge et al. 2012, Wakefield et al. 2011).

Cryodamage and lipid accumulation

One of the major problems of the in vitro produced embryos is the great sensitivity to cryopreservation. The procedure of cryopreservation of gametes and embryos can induce oxidative stress during the cycle of freeze/thaw and make cells more sensitive to ROS (Alvarez and Storey 1992). ROS can attack the lipid of the cell membrane and cause spatial modifications in membrane structure which results in cryodamage. This may explain, in part, the observed harmful consequences of cryopreservation on gamete and embryo. From another point of view, the reduced cryotolerance ability of the IVP embryos is highly associated with lipid accumulation in the cytoplasm (Rizos et al. 2002c). Lipid may be used by mitochondria as an energy source to increase the production of ATP that is required for compaction and blastocyst formation (Tarazona et al. 2006). However, one of the common morphological characteristics of IVP embryos compared to in vivo produced ones is darker cytoplasm (Figure 9) and reduced buoyant density as a consequence of excessive lipid accumulation (Abe et al. 2002, Gad et al. 2012). Lipids can be accumulated in the in vitro produced embryo

either by uptake from the culture medium supplemented with serum which containing several types of lipids (Ferguson and Leese 1999, McEvoy et al. 2000, McEvoy et al. 2001, Sata et al. 1999), or because of lack of embryo ability to metabolize the available lipid storage in the cytoplasm due to possible mitochondrial dysfunction under oxidative stress conditions (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007a, Barcelo-Fimbres and Seidel 2007b)

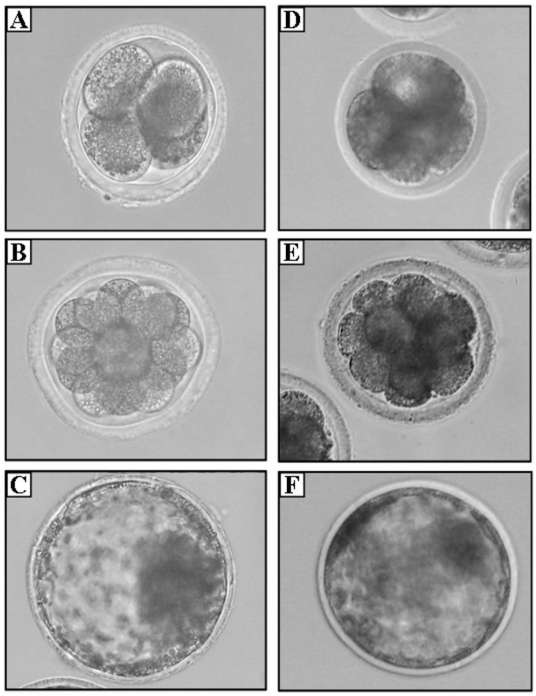


Figure 9: Morphology of bovine embryos produced in vivo (A–C) or in vitro (D–F). Images are representative of 4-cell embryos (A and D), 16-cell embryos (B and E) and blastocysts (C and F). Original magnification ×40 (Gad et al. 2012).

Apoptosis and embryo fragmentation

Among factors that have harmful effects on embryo developmental competence under in vitro culture conditions, oxidative stress is a well-known cause of cellular apoptosis as well as early embryo developmental arrest (Feugang et al. 2004, Noda et al. 1991). The imbalance between the accumulation of ROS within the cells and the antioxidants activity can accelerate programed cell death (apoptosis). Evidence suggested that increased H_2O_2 concentration could be responsible, in part, for apoptosis in mouse blastocysts (Pierce et al. 1991). In addition, direct relationship was also observed

between the elevated ROS levels and apoptosis in fragmented human embryos. Researchers concluded that the presence of DNA and cytoplasmic fragments, as a consequences of increased ROS levels, in embryos seem to be an indicator for the programed cell death in 8-cell, 16-cell and blastocyst stage embryos (Karja et al. 2004, Kitagawa et al. 2004, Yang et al. 1998). Furthermore, fragmented embryos exhibited higher H_2O_2 concentrations as compared to non-fragmented embryos. This was accompanied by higher level of cellular apoptosis only in fragmented embryos (Yang et al. 1998). It is as yet in preimplantation embryos unclear what mechanisms are involved in apoptosis as well as embryo fragmentation under oxidative stress conditions.

Similar to other cell types, mammalian preimplantation embryo apoptosis seems to be determined by the balance between the expressions of pro- and anti-apoptotic genes (Jurisicova et al. 2003, Jurisicova and Acton 2004, Liu et al. 1997, Liu et al. 2000, Spanos et al. 2002). In mammals, cell apoptosis can be triggered through two major apoptotic pathways, namely the extrinsic pathway (death receptor pathway), which occurs at the cellular membrane, or the intrinsic pathway (the mitochondrial driven pathway) which is initiated from within the cell and may be activated by several external signals (Hengartner 2000).

The extrinsic pathway is carried out by activation of death receptors of the tumour necrosis factor (TNF) superfamily, such as CD95 (APO-1/FAS) and TNF-related apoptosis-inducing ligand (TRAIL; also known as APO2L), on plasma membranes by their respective ligands (Hengartner 2000, Pitt et al. 1996, Schulze-Osthoff et al. 1998). Death ligands, such as CD95 ligand (CD95L or FASL), TRAIL/Apo-2 ligand or TNFα occupy their cognate receptors to initiate the formation of death-inducing signalling complex (DISC). The key component of DISC is the adaptor molecule FAS-associated death domain containing protein (FADD), which can bind to CD95 (Chinnaiyan et al. 1996, Kischkel et al. 1995) and interact with several proteins, including caspase proteins. Activation of caspase protease family (caspase 3, 7, 8, 9 and 10) is responsible for execution of the cell apoptosis (Boldin et al. 1996, Hengartner 2000, Muzio et al. 1996). The extrinsic pathway has been suggested to be involved in the regulation of the apoptotic pathway during early embryo development. FAS and FASL transcripts were detected in 2-cell rat embryos, 4-cell and fragmented human embryos (Kawamura et al. 2001). In addition, TRAIL mRNA and protein were detected from the 1-cell through blastocyst stage in mouse. Moreover, addition of TRAIL (5 μg/ml) and its sensitizer

(0.5 μg/ml actinomycin D) to the culture media increased the apoptotic nuclei percentage per blastocyst (8%) compared to control group (3.2%) (Riley et al. 2004). Also, TNFα mRNA was detected during early mouse embryo development (Pampfer et al. 1994). Furthermore, addition of TNFα to the culture media has been shown to induce the blastomere apoptosis of bovine embryos (Soto et al. 2003) and to delay the embryonic development in mouse (Pampfer et al. 1994). Increased levels of ROS-mediated oxidative stress have been suggested to be one of the major activators for the extrinsic pathway (Nguyen et al. 2013). For example, high levels of H_2O_2 increased apoptosis via activation of FAS-mediated apoptosis (Kasahara et al. 1997). Results of a recent study showed oxidative stress also to mediate TRAIL-induced apoptosis in HeLa cells by accumulation of ROS which led to activate caspases proteins (Nguyen et al. 2013).

The intrinsic or mitochondrial pathway is initiated by stress signals which lead to release different apoptotic factors such as cytochrome c, apoptosis inducing factor (AIF), endonuclease G and Smac/DIABLO from the mitochondria to the cytoplasm. The release of cytochrome c induces formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex, which activate caspase-9 and later caspase-3 and -7 as well as destroy the cell through DNA and proteins fragmentation (Cervinka et al. 1999, Jeong and Seol 2008, Saelens et al. 2004, Wang and Youle 2009). Moreover, AIF and endonuclease G, which have nuclease activity, can translocate to the nucleus to initiate DNA fragmentation (Bajt et al. 2006), whereas Smac/DIABLO can deactivate the inhibitors of apoptosis (IAPs) and induce a greater degree of cellular apoptosis (Hunter et al. 2007, Kohli et al. 2004, Vaux and Silke 2003, Verhagen et al. 2007)

Members of the Bcl-2 family of proteins regulate apoptosis by modulating mitochondrial outer membrane permeabilization (Chipuk and Green 2008, Harris and Thompson 2000, Kelekar and Thompson 1998). The Bcl-2 family proteins can be divided into two different major groups based on their functions. The anti-apoptotic group includes Bcl-2, Bcl-xL, Bcl-w and Mcl-1 proteins, which can block the function of pro-apoptotic proteins. And the pro-apoptotic group which can be divided into two subcategories: 1) Bok, Bax, Bak and Bik pro-apoptotic Bcl-2 family members which induce apoptosis directly via binding to outer mitochondrial membrane and increasing its permeability, leading to release of cytochrome c, AIF and endonuclease G (Lambert et al. 2004, Tripathi et al. 2012). 2) Bad, Bid and Bim pro-apoptotic Bcl-2 family

members which can indirectly regulate the pro-apoptotic function via inhibition of anti-apoptotic Bcl-2 group (Harris and Thompson 2000, Jeong and Seol 2008, Kelekar and Thompson 1998).

During early embryo development, several genes involved in intrinsic apoptosis pathway have been detected. Bcl-2 pro-apoptotic family genes Bax, Bok, Bak, Bad and Bik were constitutively expressed, being detectable in different embryo stages in human, bovine, porcine and mouse (Antczak and Van Blerkom 1999, Exley et al. 1999, Hu et al. 2012, Jurisicova et al. 1998, Jurisicova et al. 2003, Knijn et al. 2005, Metcalfe et al. 2004, Moley et al. 1998). Data of these studies suggested that embryo fragmentation may be associated with increased expression of these genes. In addition, Smac/DIABLO transcripts were detected in mouse preimplantation embryos and its protein was localized in mitochondria and found to be released into the cytosol of fragmented embryos (Honda et al. 2005). On the other hand, Bcl-2 anti apoptotic family genes such as Mc1-1, Bcl-x, are also expressed during development of human, bovine and mouse embryos (Exley et al. 1999, Jurisicova et al. 1998, Jurisicova et al. 2003, Knijn et al. 2005, Metcalfe et al. 2004). The imbalance between pro- and anti-apoptotic genes was suggested to be involved in embryo apoptosis and may be involved in early embryonic arrest in response to a suitable trigger (Jurisicova and Acton 2004, Metcalfe et al. 2004).

The intrinsic pathway can be triggered by ROS mediated oxidative stress in cellular systems (Kasahara et al. 1997, Pierce et al. 1991). Accumulation of ROS can modulate mitochondrial membrane permeability (Kroemer et al. 2007), consequently, loss of mitochondrial functions which is considered to be the starting point of intrinsic apoptosis pathway (Ferri and Kroemer 2001, Jeong and Seol 2008, Lotocki and Keane 2002). In addition, ROS mediated oxidative stress can activate the pro-apoptotic Bcl-2 family member Bax (Ho et al. 1997, Jungas et al. 2002), Bik and Bak (Maroto and PerezPolo 1997, Ritchie et al. 2009) suggesting a pro-apoptotic role of these genes in oxidative stress-induced apoptosis in mammalian cells. In mammalian embryos, a recent study has demonstrated that culturing embryos under high oxygen concentrations increased the expression levels of pro-apoptotic genes (Bax and Bid) and decreased the expression levels of the anti-apoptotic genes (Bcl-xL and Mcl-1) (Elamaran et al. 2012). This imbalance between the expression of pro- and anti apoptotic genes was accompanied by high percentage of TUNEL-positive (apoptotic) cells. On the other

hand, the expression trend of pro- and anti-apoptotic genes was reversed by decreasing the oxygen concentrations in the culture atmosphere and addition of antioxidants to the culture media (Elamaran et al. 2012).

In general, oxidative stress has recently been shown to play an important role in the initiation and progression of apoptosis via both extrinsic and intrinsic pathways in different mammalian cell types as well as in embryos (Elamaran et al. 2012, Kumar et al. 2014, Nguyen et al. 2013, Rashid et al. 2013, Sarkar et al. 2011, Shimizu et al. 2014). This role is further supported by the ability of different antioxidants such as vitamin C, SOD, N-acetylcysteine (NAC), sodium selenite and cysteamine to prevent apoptosis (Aggarwal et al. 2010, Elamaran et al. 2012, Hu et al. 2012, Saito et al. 2004, Uhm et al. 2007). In addition, the antioxidant power of Bcl-2, a potent inhibitor of apoptosis, and its ability to neutralize the detrimental effects of the ROS to cell further confirm this notion (Haddad 2004).

Defective embryo development

Oxidative stress characterized by high ROS levels has been implicated in the defective early embryonic development. It is a well-known cause of cellular fragmentation and developmental arrest of IVP embryos (Kimura et al. 2010). The two-cell block phenomenon observed in mouse embryos was associated with a rise in ROS concentrations. This phenomenon was only observed under in vitro culture conditions while, no such effect was observed in in vivo embryos (Johnson and Nasr-Esfahani 1994, Nasresfahani et al. 1990b, Nasresfahani and Johnson 1991, Noda et al. 1991). Mouse embryo development of SOD1-deficient oocytes was totally arrested at the 2-cell stage under 20% oxygen level culture conditions as a result of significant increase in superoxide levels in those embryos. While, hypoxic culture with 1% O_2 vitiated the 2-cell arrest and improved the embryonic development to 4-cell stage. In addition, the blastocyst formation rates were similar to that of embryos derived from wild-type (Kimura et al. 2010). Moreover, Bedaiwy et al., (2004) demonstrated that low fertilization rate, low cleavage rate, high embryo fragmentation, defective embryonic development as well as lower pregnancy rates are associated with increased levels of ROS in day 1 embryo culture time in vitro. Results of other studies showed reduced rate of embryo development to blastocyst stage that was accompanied by increased H_2O_2

level under high oxidative stress culture conditions in different animal species. For example, increased O_2 tension in in vitro culture atmosphere dramatically reduced embryo development to blastocysts stage significantly ($P < 0.05$) from 25.8 % to 20.4 % in human (Dumoulin et al. 1999), from 38 % to 18 % in cattle (Liu and Foote 1995), from 36.3% to 22.5% in pig (Kitagawa et al. 2004), from 38% to 29% in sheep (Leoni et al. 2007), from 31.3 to 16.1% in buffalo (Elamaran et al. 2012) and decreased the percentage of hatching blastocysts to 7% in cattle (Yuan et al. 2003). In addition, exposure of oocytes, zygotes, 2- to 4-cell embryos or 9- to 16-cell embryos to different levels of H_2O_2 resulted in a significant ($P < 0.05$) dose-dependent reduction in blastocyst development. This was in conjunction with a consistent increase in either permanent embryo arrest or apoptosis in a stage-dependent manner. On the other hand culture media supplementation with polyethylene glycol–catalase reduced ROS-induced embryo arrest, resulting in a significant ($P < 0.05$) increase in blastocyst formation rates under high O_2 tension culture conditions (Bain et al. 2011).

2.2.5 Oxidative stress and gene expression patterns in IVP embryos

In addition to development, culture environment significantly altered gene expression patterns of early stage embryos. Recently, reports have revealed the alterations in gene expression of the embryos developed in vitro compared to in vivo in different mammals such as mouse and cow (Doherty et al. 2000, Lee et al. 2001, Lequarre et al. 2001, Lonergan et al. 2003b). Furthermore, gene expression patterns of in vitro produced embryos are highly affected by the presence of high oxygen tension in the culture environment of rabbit (Koerber et al. 1998), mouse (Kind et al. 2005, Rinaudo et al. 2006) and cow (Balasubramanian et al. 2007, Harvey et al. 2007) embryos, as shown in Table 2. Result of these studies showed deviations in individual gene expression associated with cell growth and apoptosis (Kind et al. 2005, Lonergan et al. 2003b), pregnancy recognition (Lonergan et al. 2003b, Michael et al. 2006, Ocon-Grove et al. 2008) and different metabolic pathways (Balasubramanian et al. 2007, Harvey et al. 2004, Kind et al. 2005, Rizos et al. 2002b). On the other hand, in preimplantation mouse embryos, global patterns of gene expression analyzed by using microarray showed that gene expression patterns of embryos generated under low (5%) oxygen concentration culture conditions were more similar to in vivo embryos compared to embryos cultured under high (20%) oxygen concentration (Rinaudo et al. 2006).

Table 2: Effect of oxygen concentration in in vitro culture atmosphere on gene expression levels of IVP embryos

Gene	Animal	Oxygen concentration		Reference
		High O_2	Low O_2	
GLUT1	Cow		**	(Balasubramanian et al. 2007, Harvey et al. 2004)
	Mouse		**	(Kind et al. 2005)
GLUT3	Mouse		**	(Kind et al. 2005)
GLUT5	Cow		**	(Balasubramanian et al. 2007)
SOX	Cow	**		(Balasubramanian et al. 2007)
G6PD	Cow	**		(Balasubramanian et al. 2007)
PRDX5	Cow		**	(Balasubramanian et al. 2007)
NADH	Cow		**	(Balasubramanian et al. 2007)
MnSOD	Cow	**		(Correa et al. 2008)
Myotrophin	Cow	**		(Harvey et al. 2007)
ND2	Rabbit	**		(Koerber et al. 1998)
VEGF	Cow		**	(Harvey et al. 2004)
	Mouse	**		(Kind et al. 2005)
Cyclophilin18	Rabbit	**		(Santos et al. 2000)
HIF1α	Buffalo		**	(Saini et al. 2014)

Table 2: Cont.

Gene	Animal	Oxygen concentration		Reference
		High O_2	Low O_2	
HIF2α	Buffalo		**	(Saini et al. 2014)
SOD-2	Buffalo	**		(Saini et al. 2014)
PRDX1	Buffalo	**		(Saini et al. 2014)
GPX-1	Buffalo	**		(Saini et al. 2014)
Caspase3	Buffalo	**		(Saini et al. 2014)
P53	Buffalo	**		(Saini et al. 2014)
Mcl-1	Buffalo		**	(Elamaran et al. 2012)
Bcl-2	Buffalo		**	(Elamaran et al. 2012)
Bax	Buffalo	**		(Elamaran et al. 2012)
Bid	Buffalo	**		(Elamaran et al. 2012)
Global gene expression	Mouse	Vitro (5%) ≠ vitro (20%)		(Rinaudo et al. 2006)

2.3 Antioxidants and embryo defenses against oxidative stress

Deleterious effects of oxidative stress on IVP embryos resulted either from overproduction of ROS or reduced clearance of ROS by antioxidant mechanisms (Davies 2000, Guerin et al. 2001). Antioxidants can be defined as "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell 1990). Therefore, antioxidants can prevent or reduce the damage caused by biochemical

reactions in which ROS are combined with and attack the other cell molecules (Betteridge 2000, Sies 1986). Different antioxidant defense mechanisms are present in both embryos and their surroundings (Al-Gubory et al. 2010). Under in vivo conditions, oocytes and embryos seem to be secured against oxidative stress by antioxidants produced by the embryo in combination with the ones that produced by the mother and present in the follicular and oviductal fluids (Gardiner and Reed 1995, Gardiner et al. 1998). However, an in vitro set up can never achieve the exact in vivo physiology. This maybe due to the presence of several external oxidative stress inducers, moreover the lack of the paternal antioxidants that can naturally defense against oxidative stress to rescue the embryos from the oxidative damage. Therefore, control of ROS levels by antioxidants seems to be an important determining factor in successful in vitro production of mammalian embryos (Agarwal et al. 2005a, Ali et al. 1993, Alvarez et al. 1996, Bedaiwy et al. 2004, Cetica et al. 2001, Liu and Keefe 2000, Nasresfahani et al. 1990b).

2.3.1 Exogenous antioxidant control of oxidative stress

During the early embryonic development, embryos can be secured from oxidative stress by antioxidants produced internally by the embryo in combination with the ones present in their surroundings (Gardiner and Reed 1995). So, exogenous antioxidant supplements during in vitro culture may be essential (Ali et al. 2003, Sudano et al. 2010). In this regard, several exogenous antioxidants include compounds of non-enzymatic as well as enzymatic antioxidants are reported to protect the in vitro cultured embryos against oxidative stress and improve the early embryonic development.

2.3.1.1 Exogenous non-enzymatic antioxidants

Non-enzymatic antioxidants widely contain vitamins (Huang et al. 2011, Rock et al. 1996, Wang et al. 2002), trace elements and chelating agents (Dashti et al. 1995, Orsi and Leese 2001), sulfurs and thiols (Alvarez and Storey 1983, Aruoma et al. 1988, de Matos et al. 2002, Takahashi et al. 1993, Zheng et al. 1988) and polyphenol compounds (Scalbert et al. 2005). They offer a condition of protection against the oxidative damage to cell components. For example, antioxidant vitamins such as vitamin E can rescue the

cell membrane by reacting with lipid radicals and ROS which are produced during lipid peroxidation process and can attack and damage the lipid membrane bilayers (Burton et al. 1985, Quinn 2004). Also, vitamin C (ascorbic acid) protects against internal oxidative damage to DNA (Fraga et al. 1991). Thus, supplementation of the commercial in vitro culture media with vitamins (such as vitamin A, B, C and E) was suggested to defend the embryo against oxidative stress and improve the embryonic development. For instance, embryo culture medium supplemented with vitamin C (50 μM) and vitamin E (400 μM) has a significant role to improve the embryo developmental rates under oxidative stress conditions in mouse (Wang et al. 2002) and pig (Hossein et al. 2007, Huang et al. 2011). Similar observations have been reported when in vitro-produced bovine embryos were co-incubated with vitamin E (Olson and Seidel 2000a). In addition to the direct action of non-enzymatic antioxidants to scavenge ROS from the cell, they act as cofactors in the regulation of the enzymatic antioxidant functions as well as have an indirect action to reduce ROS levels in the cell (Bettger 1993, Dashti et al. 1995). Therefore, in addition to vitamins, several non-enzymatic antioxidant-related compounds, such as GSH, GSH-OEt, thiols such as β-ME and cysteamine (CSH), cysteine, hypotaurine, taurine, glutamine, melatonin, chelating agents (apotransferrin, EDTA and sodium selenite), polyphenols (3,4-dihydroxyflavone, anthocyanin, quercetin, naringenin and phenolic acids) and pyruvate, which have also direct and/or indirect antioxidant functions, were used to improve the embryo developmental competence under in vitro conditions in different species, as shown in Table 3.

Table 3: Beneficial effects of non-enzymatic antioxidant supplements on the IVM, IVF and IVP embryos

Antioxidant	Animal	Effects	Reference
Vitamin A	Cow	↑BR	(Livingston et al. 2004)
	Mouse	↑MR, ↑CR & ↑BR	(Tahaei et al. 2011)
Vitamin B5	Hamster	↑BR	(McKiernan and Bavister 2000)

Table 3: Cont.

Antioxidant	Animal	Effects	Reference
Vitamin E	Cow	↑BR, ↑BCN & ↑HR	(Olson and Seidel 2000a)
	Mouse	↑BR	(Wang et al. 2002)
	Pig	↑BR &↓DNA frag.	(Hossein et al. 2007, Kitagawa et al. 2004)
GSH	Cow	↑CR &↑BR	(Luvoni et al. 1996)
	Mouse	↑BR	(Gardiner and Reed 1994)
	Pig	↑CR, ↑BR, ↑BCN, ↓AC &↓DNA frag.	(Bhandari et al. 2007, Li et al. 2014)
GSH-OEt	Cow	↑BCN	(Curnow et al. 2010)
	Macaque	↑MR, ↑FR, & ↑DR	(Curnow et al. 2010, Curnow et al. 2011)
β-ME	Cow	↑BR	(de Matos et al. 1996, Takahashi et al. 2002)
	Pig	↑BR, ↑BCN, ↓AC &↓DNA frag.	(Kitagawa et al. 2004, Yuh et al. 2010)
Cysteamine	Cow	↑BR &↑HR	(de Matos et al. 1996, de Matos et al. 2002)
	Buffalo	↑CR, ↑DR, &↑BR	(Anand et al. 2008, Prasad et al. 2013)
	Goat	↑CR &↑BR	(De et al. 2011)
	Mouse	↑MR, ↑FR &↑BR	(Roushandeh et al. 2007)
	Pig	↑FR, ↑DR, &↑BR	(Grupen et al. 1995)
Cysteine	Cow	↑BR	(Ali et al. 2003, de Matos et al. 1996)
	Pig	↑CR, ↑BR, ↑BCN, ↓AC &↓DNA frag.	(Katayama et al. 2007, Li et al. 2014)

Table 3: Cont.

Antioxidant	Animal	Effects	Reference
Hypotaurine	Cow	↑BR &↑BCN	(Fujitani et al. 1997, Guyader-Joly et al. 1998)
	Hamster	↑DR &↑BR	(Barnett and Bavister 1992)
Taurine	Cow	↑BR	(Liu and Foote 1995)
	Buffalo	↑BR	(Manjunatha et al. 2009)
	Mouse	↑BR &↑BCN	(Dumoulin et al. 1992)
	Rabbit	↑BR &↑BCN	(Li et al. 1993)
Glutamine	Mouse	↑DR &↑HR	(Lane and Gardner 1997)
Melatonin	Cow	↑BR	(Papis et al. 2007)
	Buffalo	↑BR	(Manjunatha et al. 2009)
	Mouse	↑FR, ↑DR &↑BR	(Ishizuka et al. 2000)
	Sheep	↑HR	(Abecia et al. 2002)
Penicillamine and epinephrine	Cow	↑CR, ↑DR &↑BR	(Miller et al. 1994)
Apotransferrin	Cow	↑CR &↑BR	(Choi et al. 2006)
	Mouse	↑DR &↑BR	(Nasresfahani and Johnson 1992)
EDTA	Cow	↑BR	(Olson and Seidel 2000b)
	Mouse	↑BR	(Nasresfahani et al. 1990a, Orsi and Leese 2001)
	Pig	↑CR, ↑BR, ↑BCN &↓AC	(Kim et al. 2006)
Sodium selenite	Mouse	↑FR, ↑CR, ↑DR, ↑BR &↑HR	(Abedelahi et al. 2010)
	Pig	↑BR, ↑BCN &↓AC	(Uhm et al. 2007)

Table 3: Cont.

Antioxidant	Animal	Effects	Reference
Polyphenols	Cow	↑BR, ↑BCN &↓AC	(Lee et al. 2011, Wang et al. 2007)
	Pig	↑BR	(You et al. 2010)
Pyruvate	Cow	↑DR, ↑BR &↑HR	(Morales et al. 1999)

MR, maturation rate; CR, cleavage rate; FR, Fertilization rate; DR, Developmental rates from 2-cell to morula stage; BR, Blastocyst rate; BCN, Blastocyst cell number; AC, apoptotic cell number; HR, hatching rate; DNA frag., DNA fragmentation

2.3.1.2 Exogenous enzymatic antioxidants

In addition to non-enzymatic compounds, the antioxidants that can be supplemented to embryo culture media can include a group with an enzymatic action, such as SOD family, CAT, TXN, GPX. Utilization of those antioxidant enzymes from the culture media is effective for improving the in vitro embryo development in different animal species. For example, culture media supplementation with SOD reduced ROS levels and improved the bovine embryo development (blastocyst rate with 0, 300 and 600 U/ml of SOD, respectively, was 26%, 26% and 30%) (Liu and Foote 1995). In porcine, culturing embryos in the presence of SOD increased the development rate to the blastocyst stage, improved the embryo quality by increasing the total cells number and decreasing apoptotic cells significantly ($P < 0.05$) at blastocyst stage compared to control without supplementation (Yuh et al. 2010). Similar effects were also observed for SOD in rabbit (Li et al. 1993) and mouse embryos (Nonogaki et al. 1992). Also, addition of free radical scavenger enzyme TXN to the in vitro culture media (0.5 µg/ml) dramatically promoted a higher ($P<0.05$) frequency of cleavage of the bovine embryos (Cleavage rate with 0, 0.1, 0.5 and 1 µg/ml of SOD was 55.6 ± 4.4, 64.4 ± 8.9, 81.9 ± 9.5 and 69.9 ±10.0 % respectively), improved the embryo development to blastocyst stage in vitro under 5% O_2 culture condition (blastocyst rate with 0, 0.1, 0.5 and 1 µg/ml of SOD was 18.9 ± 5.8, 25.6 ± 8.1, 34.0 ± 8.6 and 32.3 ± 4.8 % respectively) and serving embryos a protection from oxidative damage under in vitro culture conditions (Bing et al. 2003). Similar improvement in developing rates were obtained in porcine embryos by adding

1.0 mg/ml TXN (Ozawa et al. 2006) and in mouse embryos using concentration of 500 μg/ml of copper-zinc SOD or 500 μg/ml of TXN (Nonogaki et al. 1991). In another study, in addition to SOD (100-7,000 U/ml), beneficial effects of antioxidants on blastocyst and hatching rates were reported for murine zygotes cultured under 5% oxygen level in the presence of catalase (50-100 U/ml) (Orsi and Leese 2001). Moreover, SOD and TXN antioxidant enzymes were able to enhance the embryonic development in mouse by overcoming the mouse 2-cell block under vitro conditions which is believed to be due to oxidative stress (Natsuyama et al. 1993, Noda et al. 1991).

2.3.2 Endogenous antioxidant control of oxidative stress

Antioxidant control of ROS production and propagation in embryo is highly complex. Embryos, as any biological systems, have evolved a variety of internal antioxidant defenses to control ROS levels and enable them to survive under oxidative stress environments. These antioxidant defenses include mechanisms of non-enzymatic action ,such as GSH (Gardiner and Reed 1994, Takahashi et al. 1993) and enzymatic action such as SDOs, GPX and CAT (El Mouatassim et al. 1999, Harvey et al. 1995, Takahashi 2012). It is evident that the antioxidant enzymes are working together in different antioxidant systems, such as sirtuin 3 (Sirt3) and NRF2-KEAP1 systems, to protect mammalian cell against oxidative stress. In embryos, a recent study has identified Sirt3 as an endogenous protective factor against oxidative stress during preimplantation development under IVF and in IVC conditions (Kawamura et al. 2010). Sirt3 induced the activity of the antioxidant enzyme SOD2, which is considered being a critical antioxidant enzyme in the mitochondrial matrix, resulting in enhancement of scavenging of ROS (Qiu et al. 2010, Tao et al. 2010). Another very important antioxidant mechanism against oxidative stress is the NRF2-mediated oxidative stress response pathway. During embryonic development, activity of NRF2-mediatet oxidative stress response and its downstream antioxidants, such as SOD1, CAT and TXN1 were observed in bovine (Gad et al. 2012, Held et al. 2012) and mouse (Dong et al. 2008, Harris and Hansen 2012, Leung et al. 2003). Although NRF2-mediatet oxidative stress response pathway is previously considered as one of the most powerful antioxidant systems in other mammalian cells (Ade et al. 2009, Brown et al. 2010,

Chanas et al. 2002, Dhakshinamoorthy and Jaiswal 2001, Dreger et al. 2009, Enomoto et al. 2001, Giudice et al. 2010, Kim and Vaziri 2009, Knorr-Wittmann et al. 2005, Li et al. 2010), very few information are available about the activity of NRF2 antioxidant pathway in the embryo.

2.4 NRF2-mediated oxidative stress response

One of the major mechanisms in the cellular defense against oxidative stress is activation of the Nrf2-antioxidant response element signaling pathway. NRF2 is a key transcription factor which belongs to the cnc ("cap 'n' collar") subfamily of the basic region leucine zipper transcription factors (Chan et al. 1996, Giudice et al. 2010). Recently, several investigators have described the vital role of NRF2 in restoring the balance between generation of oxidants and activity of antioxidants in the animal cells under oxidative stress conditions. There is considerable experimental evidence suggesting that NRF2 can regulate the transcription activity of different cytoprotective antioxidant and detoxification enzymes via the NRF2-KEAP1 cytoplasmic oxidative stress system (Chanas et al. 2002, Chen and Kunsch 2004, Chen et al. 2006, Chen et al. 2012, Chen et al. 2014, Itoh et al. 1999, Itoh et al. 2003, Kobayashi and Yamamoto 2005, Nguyen et al. 2003, Nguyen et al. 2009, Villeneuve et al. 2009). NRF2 downstream genes are multifunctional and encode antioxidant proteins, phase 2 detoxifying enzymes, transcription factors, glutathione biosynthesis enzymes, chaperone proteins and scavenger receptors (reviewed by Kobayashi and Yamamoto 2005). NRF2 protein contains six Neh (NRF2-ECH homology) domains. Within each domain, particular characteristics related to specific functions have been identified. NRF2 Neh1 domain contains the DNA binding site and aids to heterodimerize with small MAF proteins for starting the transactivation action of NRF2. KEAP1 binding site is located in the Neh2 domain. Transactivation activities of NRF2-sMAF complex are promoted by NRF2 Neh3, Neh4 and Neh5 domains. The degron sequence of NRF2 protein, that directs the starting site of degradation, is located in Neh6 (Itoh et al. 1995, Itoh et al. 1999, Kobayashi et al. 2002).

2.4.1 NRF2-KEAP1 antioxidant system

Under normal oxidative stress free conditions, the cytoplasmic inhibitor KEAP1 is located and attached with NRF2 within the cytoplasm and targeting it for ubiquitination and proteasomal degradation (Figure 10A). Accordingly, KEAP1 retains only low basal level of NRF2 in cytoplasm to mediate the constitutive expression of NRF2 downstream genes (Giudice et al. 2010, Zhang 2006). Under oxidative stress conditions, in response to intracellular redox environment changes, a signal including phosphorylation and/or redox modification of vital cysteine residues in KEAP1 reduces the KEAP1–Cullin 3 (CUL3)-Ring box 1(RBX1)-Ubiquitin conjugating enzyme (E3) complex activity, which results in dissociation of the attachment between KEAP1 and NRF2 as well as releasing of NRF2, allowing it to be free in cytoplasm. As consequence, free NRF2 protein translocates into the nucleus and combines with other transcription factors, such as sMaf, ATF4, JunD and PMF-1, in order to activate the antioxidant response elements (AREs) of many cytoprotective genes (glutathione reductase (GSR), superoxide dismutase family (SOD1, 2 and 3), CAT, TXN1, GPX-1 and 3 and NRF2 itself, as shown in Figure 10B (Lau et al. 2008, Nguyen et al. 2003, Osburn and Kensler 2008). After cellular redox homeostasis recovery, KEAP1 travels into the nucleus and separates NRF2 from the ARE, consequently, NRF2/ARE signaling pathway breakdown. NRF2–KEAP1 complex translocates then out of the nucleus by the nuclear export sequence (NES) in KEAP1. Afterwards, NRF2–KEAP1 complex attaches with the Cul3-RBX1-E3 core ubiquitin machinery again resulting in NRF2 degradation, as shown in Figure 10C (Dong et al. 2008, Giudice et al. 2010).

2.4.2 NRF2-mediated oxidative stress response and lipid metabolism

In addition to the role of NRF2 in the cellular defense against oxidative stress, recent experiments showed that NRF2 activity is in relation with the lipid metabolism. And it has been concluded that NRF2 may play a role in lipid accumulation in mouse liver via regulation of fatty acid metabolism genes (FAs, FAE, ACC, SCD, AOX, Cpt1, SREBP) and cholesterol biosynthesis genes, such as HMG-CoA synthase and HMG-CoA reductase (Huang et al. 2010, Okada et al. 2009, Tanaka et al. 2008). In bovine embryos, series of experiments has been done to explore the transcriptome profiles of

bovine embryos at blastocyst stage which were generated under alternative in vivo and/or in vitro culture conditions during different time points of early embryo development. Functional classification of DEG's showed that lipid metabolism was the most affected molecular mechanism by the culture environment in the in vitro generated blastocysts. Results showed down-regulation in the expression of genes involved in steroids metabolism (CYP11A1, HSD3B1 and APOA1), cholesterol metabolism (CYP11A1 and HSD17B7), lipid secretion, translocation and metabolism (ABCC2 and SC4MOL) compared to the in vivo control group. In the same study, pathway analysis results revealed that NRF2-mediated oxidative stress response pathway was the dominant pathway in in vivo generated blastocysts with up-regulation for most of the pathway genes, especially those genes which have antioxidant functions. However, some in vitro produced embryos showed opposite fashion of expression. These results showed a possible correlation between the expression patterns of genes involved in lipid metabolism and NRF2-mediated oxidative stress response pathway and it has been concluded that NRF2 antioxidant response pathway has a vital role to play in embryo metabolism during early bovine embryonic development (Gad et al. 2012).

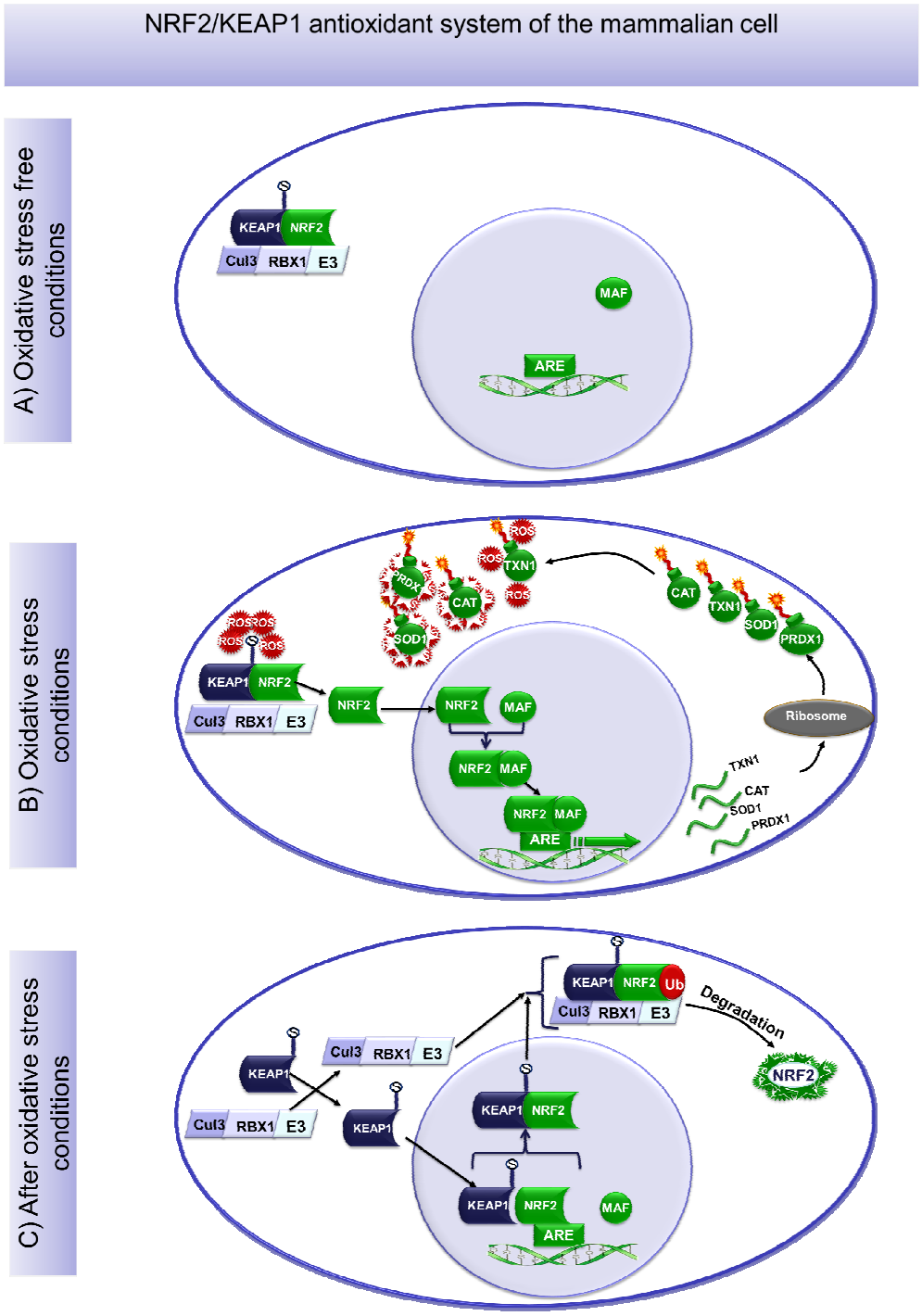


Figure 10: NRF2-KEAP1 antioxidant mechanism under oxidative stress free conditions (A), oxidative stress conditions (B) and after oxidative stress conditions (C).

3 Materials and methods

In order to examine the ability of bovine embryos to activate NRF2 mediated oxidative stress response pathway under in vitro culture conditions and its association with embryo survival and metabolism, two experiments were preformed as following:

Experiment 1: Expression analysis of NRF2 mediated oxidative stress response and lipid metabolism pathway genes in bovine embryos cultured in vitro under oxidative stress conditions.

In this experiment, as indicated in the experimental design (Figure 11), in order to elucidate whether the bovine embryos at the early developmental stages are able to activate NRF2 and its downstream antioxidant-related genes as a response to oxidative stress, embryos were cultured in vitro under low (5%) oxygen (similar to in vivo oviduct oxygen level) or high (20%) oxygen level as commonly used in standard in vitro culture system. Embryos from 2-, 4-, 8-, 16-cell and blastocyst stages were collected respectively at 32, 48, 72, 92 and 168-192 hours post-insemination (hpi) from both oxygen level culture conditions. The expression of NRF2 pathway genes including antioxidants (CAT, HMOX1, NQO1, PRDX1, SOD1 and TXN1), NRF2 cytoplasmic inhibitor (KEAP1) and genes involved in lipid metabolism namely: sterol regulatory element binding protein transcription factor1 (SREBP1), acetyl-CoA carboxylase alpha (ACACA1), peroxisome proliferator-activated receptor alpha (PPARAα) and carnitine palmitoyl transferase-2 (CPT2) were quantified in several stages of development. In addition, protein of NRF2 and KEAP1 gene was determined at blastocyst stage. Moreover, the ROS level, mitochondrial activity and lipid content phenotypes were evaluated at blastocyst stage.

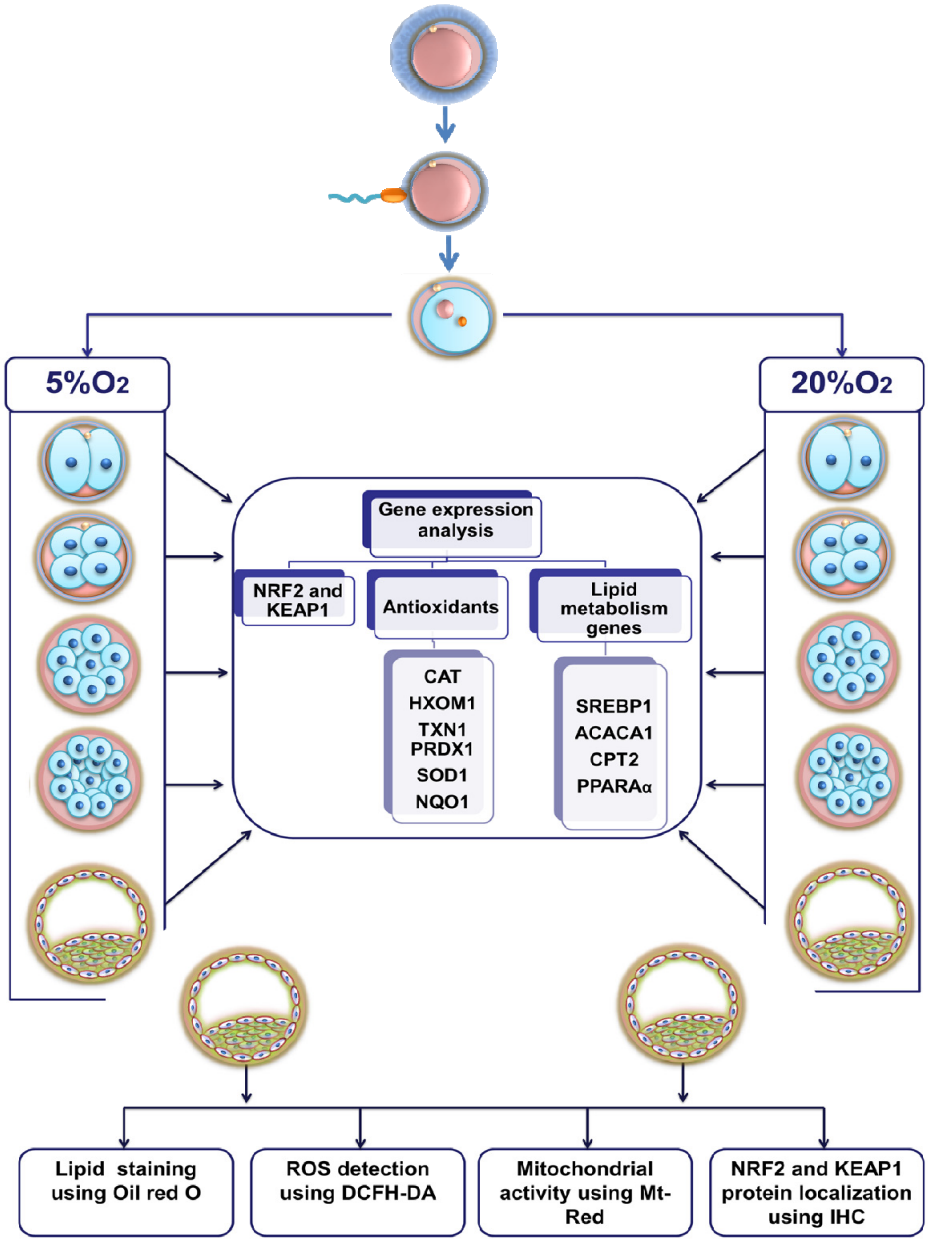


Figure 11: Overview of experiment 1. After IVM and IVF of the collected oocytes, two groups of embryos were cultured under 5% and 20% O_2. Samples from each O_2 culture condition were collected at 2-, 4-, 8-, 16-cell and blastocyst stage of development for expression analysis of NRF2, KEAP1, antioxidants and lipid metabolism genes. In addition, blastocysts from each group were used for assessment of NRF2 and KEAP1 proteins, ROS level, mitochondrial activity and lipid content.

Experiment 2: Association of NRF2-mediated oxidative stress response pathway activity with the developmental competence of bovine in vitro produced embryos.

To determine whether the ability of early bovine embryos to tolerate high oxidative stress under in vitro culture conditions is associated with their NRF2 activity, as shown in Figure 12, NRF2 mediated oxidative stress response pathway related genes were analysed in developmentally competent vs. incompetent 2-cell and blastocyst stage embryos derived from high or low oxidative stress conditions. For this, the developmental pattern of the embryos was examined at 27, 30, 32, 34, 36, 40 and 42 hpi. 2-cell stage embryos obtained before 32 hpi were defined as early cleaving embryos and considered as developmentally competent embryos, while 2-cell embryos obtained after 36 hpi were defined as late cleaving embryos and considered as developmentally incompetent embryos. Similarly, the development of blastocyst stage embryos was evaluated at day 7 and 8 post-insemination (pi). Day 7 pi blastocysts (D7 pi) were considered as competent embryos, while, those which reached to the same stage at day 8 pi (D8 pi) were categorized as incompetent as evidenced by Hasler et al. (1995) and Bernardi & Delouis (1996). 2-cell and blastocyst stage embryos from each oxygen level (5% and 20%) culture environment and competence category (competent and incompetent) were used to investigate the expression of NRF2, KEAP1, NRF2 downstream antioxidant genes (CAT, PRDX1, SOD1 and TXN1) and genes related to lipid metabolism (SREBP1 and CPT2) by using q-PCR. Moreover, the NRF2 transcriptional activity in competent vs. incompetent embryos was assessed by evaluation of the amount and distribution of NRF2 and KEAP1 proteins using immunofluorescence technique. Furthermore, the level of ROS, mitochondrial activity and lipid content were determined for embryos from each tested category. Finally, the ability of the competent and incompetent blastocyst embryos to survive after cryopreservation (in terms of hatching rates) was evaluated.

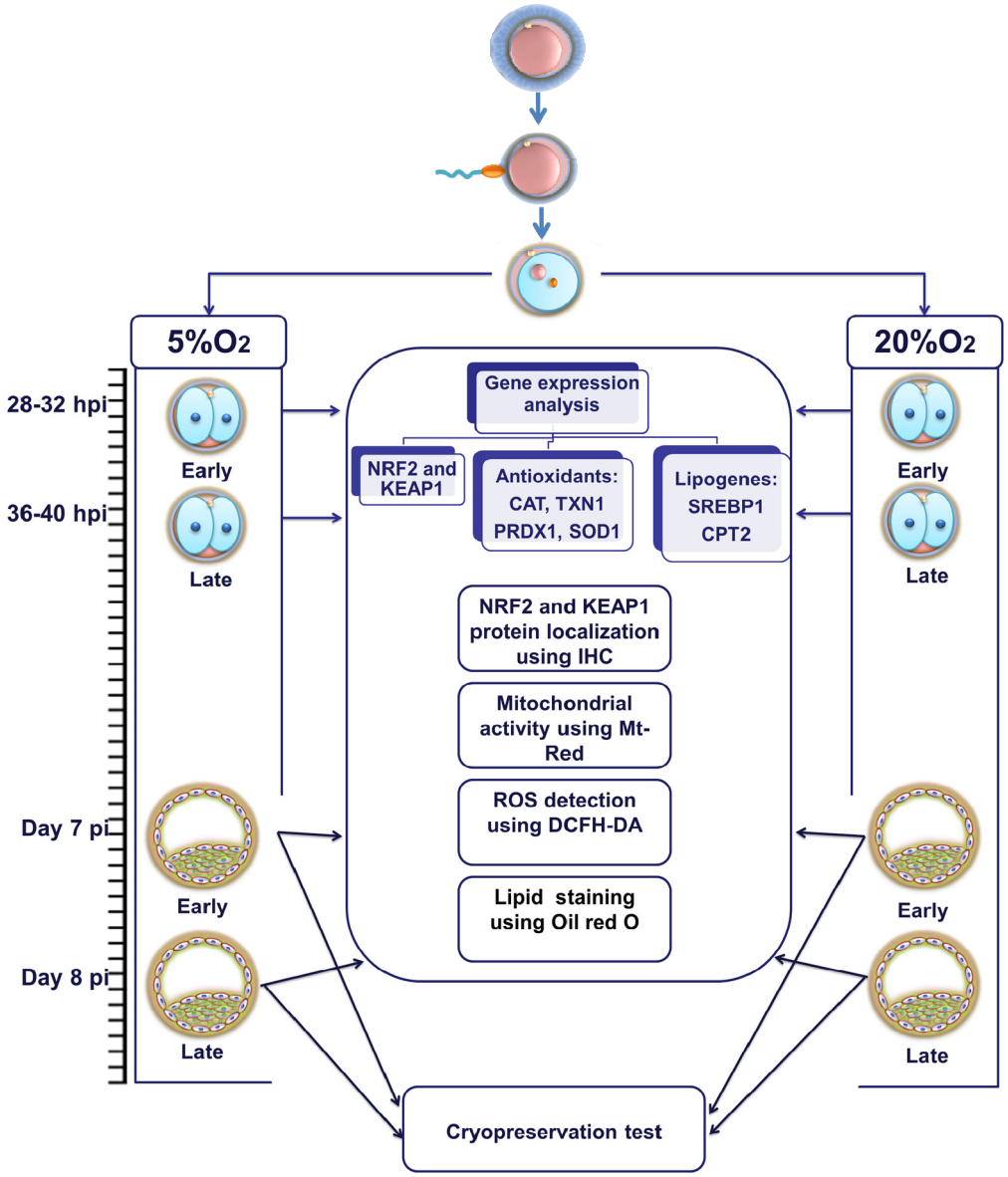


Figure 12: Overview of experiment 2. After IVM and IVF of the collected oocytes, two groups of embryos were cultured under 5% and 20% O_2. Early competent 2-cell and blastocyst stage embryos were collected before 32 hpi and at D7 pi respectively while, late incompetent 2-cell and blastocyst stage embryos were collected after 36 hpi and at D8 pi respectively. Embryos from each category were used for expression analysis of NRF2, KEAP1, antioxidants and lipid metabolism genes and for evaluation of NRF2 and KEAP1 proteins, ROS level, mitochondrial activity and lipid content. Finally, cryopreservation test was preformed for D7 pi and D8 pi blastocysts.

3.1 Materials

3.1.1 Samples

Bovine ovaries were collected from a commercial slaughterhouse and transported to the laboratory in 37°C saline solution within 3 hours time. The ovaries were then washed twice in fresh phosphate buffer saline (PBS). Cumulus oocyte complexes (COCs) were then aspirated from follicles with 2–8 mm size. Only COCs with a homogenous cytoplasm and surrounded by at least three layers of compact cumulus cells were used for in vitro maturation, in vitro fertilization and in vitro embryo production.

3.1.2 Equipment, chemicals, kits, biological and other materials

In the present study, several items of equipment, chemicals, kits, reagents and media formulation were used according to the manufacturer's instructions.

3.1.2.1 Items of equipment

Equipment	Manufacturer
ABI PRISM® 7000 SDS	Applied Bio systems Foster City, CA, USA
Gel documentation system XR	Bio-Rad Laboratories, CA, USA
My Cycler Thermal cycler Electrophoresis	BioRad, Munich, Germany
Freezer (-20°C)/Antibacterial system	Bosch, Germany
Confocal laser scanning microscope, CLSM LSM-780	Carl Zeiss, Germany
Tuttnauer autoclave	Connections unlimited, Wettenberg, Germany
Shaker	Edmund Bühler, Tübingen, Germany
Memmert CO_2 incubator	Fisher Scientific, Leicestershire, UK

Micropipette PIPETMAN Neo from 0.2–2 µl to 100–1000 µl	Gilson S.A.S, Villiers-le-Bel, France
Pipette tip, Ultratip Gelson style 200 µl and 1000 µl	Greiner bio-one, Kremsmünster, Austria
Serological Pipette, 1 ml, 2 ml, 5 ml and 10 ml	Greiner bio-one, Kremsmünster, Austria
HEAR safe Bio-flow safety hood	Heraeus instruments, Meckenheim, Germany
Centrifuge Hermel Z200 M/H, Z233 and Z300	Hermle, Wehingen, Germany
Magnetic stirrer	IKA-Werke GmbH & Co. KG, Staufen, Germany
Digital balance KERN EMB1200-1	KERN & SOHN, Balingen, Germany
Digital pH meter	Knick, Berlin, Germany
Inverted fluorescence microscope DM IRB	Leica, Bensheim, Germany.
Refrigerator (4°C)/Antibacterial system	Liebherr
Microwave Micromaxx MM 41580	Medion Electronics,Swindon, Wiltshire, UK
Digital balance	Mettler Toledo, Switzerland
Epifluorescent microscope	Olympus, Tokyo, Japan
Quick rack tip transfer system DNase/RNase free Tips	Pyrogens, south west, Utah, USA
Glass cover slips	Roth , Karlsruhe, Germany
Ultra low temperature freezer (-80°C)	Sanyo electric Co.,Ltd, Japan
Ice maker	SCOTSMAN, Milan, Italy
Microplates for real-time PCR	STARLAB GmbH (Ahrensburg)
Nanodrop 8000 Spectrophotometer	Thermo Fisher Scientific, DE, USA

Four and 24 well dishes,	Thermo Fisher Scientific, Nunc, Roskilde, Denmark
Pipettor tip Maxymum Recovery 0.5 to 10µL	Thermo Fisher Scientific, Nunc, Roskilde, Denmark
Incubated/Refrigerated Stackable Shaker Max Q 6000	Thermo scientific, IWA, USA
Diagnostic microscope slides	Thermo scientific, IWA, USA
CEQ^{TM} 8000 Genetic Analysis sequencing machine	Beckman Coulter, Krefeld, Germany

3.1.2.2 Chemicals, kits and biological materials

The following list of chemicals and other biological materials were used in the present study:

Manufacturer/Supplier	Chemicals and biological materials
Arcturs, CA, USA.	PicoPureTM RNA isolation kit
Beckman Coulter, Krefeld, Germany	Sample loading solution (SLS)
	Dye terminator cycle sequencing (DTCS)
	Glycogen for sequencing
Bio-Rad laboratories, Munich, Germany	iTaq SYBR Green Supermix with ROX
Burlingame, CA, USA	4',6'-diamidino-2-phenylindole hydrochloride(DAPI)
Gibco BRL, life technologies, Karlsruhe, Germany	Essential amino acids (BME)
	Non essential amino acids (MEM)
Invitrogen Life Technologies, Karlsruhe, Germany	DTT
	5x First-Stand buffer
Invitrogen, Carlsbad, CA, USA	Superscript II reverse transcriptase
	Hoechst 33342
	MitoTracker® Red CMXRos
	H2DCFDA fluorescent probe

Lifespan Biosciences, WA, USA	Fluorescein isothiocyanate (FITC) Conjugated goat anti-rabbit secondary antibody
Primers MWG Biotech, Eberberg, Germany	Oligonucleotide
Promega, WI, USA	Oligo (dT) 25 primer 10x PCR buffer 2x rapid ligation buffer Hemi-calcium lactate pGEM®-T vector Random primer Ribo-nuclease inhibitor (RNasin) T4 DNA ligase Bovine serum albumin (BSA) RNase-free DNase
Quigen, Hiden, Germany	QIAquick PCR Purification kit
Roche Diagnostics GmbH, Mannheim, Germany	Triton X-100
Roth , Karlsruhe, Germany	Ampicillin Boric acid Chloroform dNTPs Dimethyl sulfoxide (DMSO) Disodium hydrogen phosphate Ethylenediaminetetra acetic acid Glycin Potassium chloride Potassium dihydrogen phosphate Pepton Sodium chloride Tris Acetic acid ($C_2H_4O_2$) Agar-Agar

	Ethanol
	Ethidium bromide
	Hydrochloric acid
	Igepal
	Isopropyl β-D-thiogalactoside (IPTG)
	X-Gal (5-bromo-4-chloro-3-indolylbeta-Dgalactopyranoside)
Santa Cruz Biotechnologies Inc., CA., USA	Rabbit polyclonal primary antibody against NRF2
	Rabbit polyclonal primary antibody against KEAP1
Sigma-Aldrich Chemie GmbH, Munich, Germany	Agarose
	Calcium chloride
	Formaldehyde
	Hepes
	Hydroxylamine
	L-Glutamine
	Magnesium chloride
	Mineral oil
	Donkey serum
	Polyvinyl alcohol (PVA)
	Phenol red solution (5% in D-PBS)
	TCM199 medium
Sigma-Aldrich Chemie GmbH, Taufkirchen	Penicillin
	Hypotaurin
Sigma-Aldrich Inc, MO, USA	Sodium hydrogen carbonate
	Streptomycin sulphate
	Sodium pyruvate
	Taq DNA polymerase
Sigma-Aldrich, Munich, Germany	Oil red O
Stratagene, Amsterdam, Neatherland	*E. coli* competent cells

3.1.2.3 Media, buffers and other reagents

Media, buffers and other reagents and its constituents that were used in the present study have been listed as following:

Maturation medium: Modified Parker Medium (MPM-110 ml):	Sodium hydrogen carbonate	0.080 g
	Hepes	0.140 g
	Sodium pyruvate	0.025 g
	L-Glutamin	0.010 g
	Streptomycin phosphate	0.003 g
	Penicillin	0.002 g
	Medium	199 99.0 ml
	Hemicalcium lactate	0.060 g
	Water added to	110.0 ml
Fertilization medium: Fert-TALP (50 ml)	Sodium chloride	0.3300 g
	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0021 g
	Penicillin G	0.0032 g
	Magnesium chloride	0.0050 g
	Calcium chloride	0.0150 g
	Sodium lactate solution	93 µl
	Phenol red solution (5 % in DPBS)	100 µl
	Water added to	50 ml
Culture medium CR1-aa (50 ml)	Hemi-calcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	L-Glutamine	0.0073 g
	Phenol red solution (5% in DPBS)	100 µl

LB-agar	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH2O added to	800.0 ml
LB-broth	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH2O added to	800.0 ml
Lysis buffer (100 µl)	Igepal (0.8%)	0.8 µl
	RNasin	5 µl
	DTT	5 µl
	ddH2O added to	100 µ
Tris-acetate-EDTA buffer	Tris	242.0 mg
(TAE 50x), pH 8.0	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml
	ddH2O added to	1000.0 ml
Tris-EDTA (TE 1x) buffer	Tris (1 M)	10.0 ml
	EDTA (0.5 M)	2.0 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH2O added to	25 ml
X-gal	X-gal	50 mg
	ddH2O added to	1000 ml
IPTG (0.5 M):	IPTG	000 1.2 g
	Water added to	10 ml
PBS 1X (PH 7.4)	Sodium chloride	8 g
	Potassium chloride	0.20 g
	Disodium hydrogen phosphate	1.44 g
	Potassium dihydrogen phosphate	0.24 g

DEPC-treated water (1000 ml)	DEPC	1 ml
	ddH2O	1000 ml
Para formaldehyde 16% (PFA) 10 ml	N, N'-dimethylformamide	1.0 ml
	Para formaldehyde	1.6 g
	added to water	10 ml
PFA (4%)	PFA (16%)	250 µl
	PBS 1X	750 µl
PBS+PVA (0.3%)	Polyvinyl alcohol (PVA)	150 mg
	PBS 1X	50 ml
Donkey serum (3%)	Donkey serum 100%	3 ml
BSA (3%)	BSA	0.15 g
	added to PBS+PVA	5 ml
Glycin-PBS 30 mmol	Glycin	0.022 g
	PBS+PVA	10 ml
Glycin-PBS 0.3 mmol	Glycin-PBS 30 mmol	100 µl
	PBS+PVA	9.9 ml
Triton (0.5%)	Triton X-100	25 µl
	Glycin-PBS 0.3 mmol	5 ml
Physiological saline solution	Sodium chloride	9 g
	added to water	1000 ml
dNTP solution	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	ddH2O added to	400.0 µl

3.1.2.4 Programs and statistical packages used

In the present study, the following list of computer programs was used:

Program	Source of the program
BLAST program	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Image Lab 2	Bio-Rad Laboratories, CA, USA
Primer 3 (version 4)	http://frodo.wi.mit.edu/primer3/

Prism for windows (ver.5.0)	GraphPad software, Inc.
ZEN lite 2011	Carl Zeiss International

3.2 Methods

3.2.1 IVP of bovine embryos under oxidative stress conditions

In vitro maturation of oocyte

COCs were aspirated from ovaries obtained from slaughterhouse using a sterilized 10 ml syringe attached to an 18 G needle. The collected oocytes were then categorized under the microscope and only COCs with a homogenous cytoplasm and surrounded by at least three layers of compact cumulus cells were used for in vitro maturation. COCs were then transferred (in a group of 50 oocytes/well) to modified TCM199 (Sigma-Aldrich, Munich, Germany) culture media supplemented with 4.4 mM HEPES, 33.9 mM NaCHO3, 2 mM pyruvate, 2.9 mM calcium lactate, 55 mg/ml gentamicin and 12% (v/v) heat-inactivated oestrus cow serum (OCS). In an incubator, at a temperature of 39 °C and under atmospheric oxygen concentration (20%), IVM was performed for 22-24 hours.

In vitro fertilization

Following in vitro maturation, IVF was performed in Fert-TALP medium supplemented with 20 mM penicillinamine, 10 mM hypotaurine, 2 mM noradrenaline, 6 mg/ml BSA, 50 mg/ml gentamicin and 1 mg/ml heparin (Parrish et al. 1986, Parrish et al. 1988). Frozen-thawed bull sperms with a concentration of $2*10^6$ sperms/ml were used for in vitro fertilization which is initiated by co-culture of sperms and COCs for 20 hours. IVF procedures were performed at a 39°C temperature and an atmosphere of 20% oxygen level.

In vitro culture of embryos under oxidative stress conditions

Presumptive zygotes were transferred to CR-1aa culture medium supplemented with 10% OCS, 10 ml/ml BME (essential amino acids) and 10 ml/ml MEM or non-essential amino acids (Rosenkrans and First 1994). Zygotes were cultured either under 5% or 20% oxygen levels as a source of oxidative stress until day 8 (blastocyst stage) at 39°C. Embryos at 2-, 4-, 8-, 16-cell and blastocyst stage were harvested at 32, 48, 72, 92 and 168-192 hpi, respectively. For gene expression analysis, the collected embryos at each developmental stage were then washed twice in PBS and snap frozen in cryo-tubes containing lysis buffer [40 U ml-1 RNasin (Promega, WI, USA), 0.8% Igepal (Sigma-Aldrich, MO, USA), 5 mM dithiothreitol (DTT) (Promega, WI,USA)]. All the samples were then stored at -80°C until RNA isolation.

3.2.2 Monitoring embryo development under oxidative stress conditions

In order to investigate the effect of elevated oxygen level in the culture atmosphere conditions on the early bovine embryo development in terms of cleavage and blastocyst rates, the embryo development was assessed by determining the cleavage rate at 24-40 hpi and the blastocyst rate at days 7 (168 hpi) and 8 (192 hpi) either under low oxygen concentration, as presented in oviduct (5%), which was considered as a control group, and under high oxygen level (20%) which was considered as a source of oxidative stress to the in vitro cultured embryos.

3.2.3 Gene expression analysis of NRF2 pathway and lipid metabolism related genes

3.2.3.1 Total RNA isolation from in vitro produced embryos

RNA was isolated from pooled frozen bovine embryos of three biological replicates each containing fifty 2-cell, thirty 4-cell, twenty 8-cell, fifteen 16-cell and ten blastocyst stage embryos from each oxygen level culture conditions. Total RNA isolation and purification were performed using PicoPure RNA isolation kit (Arcturs, Munich, Germany) according to the manufacturer's instructions. For this, 70 µl extraction buffer was added to each biological replicate and mixed well by repeated pipetting followed by

incubation for 30 min in preheated incubator at 42°C. Before the end of incubation time, the purification columns were prepared by incubating of 250 µl conditioning buffer inside the columns at room temperature for 5 min and centrifuging them at 13250 rpm for 1 min. Next, one volume of 70% ethanol (70 µl) was added to each sample at the end of the incubation time. The samples were then transferred to the pre-conditioned purification columns and centrifuged at 1000 rpm for 2 min to bind the RNA into the columns and immediately followed by a centrifugation at 13500 rpm for 30 s to remove any ethanol residues. After that, the samples were washed again by adding 100 µl washing buffer 1 into the column and centrifuging at 9475 rpm for 1 min. To purify the isolated RNA from any DNA contamination, on column, DNA digestion was preformed using DNase solution (Qiagen GmbH, Hilden, Germany). Total RNA was then washed once with washing buffer 1 (40 µl) and twice with washing buffer 2 (100 µl). Finally, RNA was eluted in 11 µl elution buffer and stored at -80°C until further use.

3.2.3.2 cDNA synthesis from total RNA

Equal amount of RNA input (200ng) was used for cDNA synthesis from each group of embryos derived either from 5% or 20% oxygen level culture conditions. cDNA synthesis was performed using oligo (dT) 25 and random primer (Promega Madison WI, USA) and superscript reverse transcriptase II (Invitrogen, Karlsruhe, Germany). Each reaction containing 4 µl of 5X first-strand buffer (375 mM KCl, 15 mM MgCl2, 250 mM Tris–HCl, pH 8.3), 2 µl of dithiothreitol (Promega Madison WI, USA), 1 µl dNTP, 0.7 µl of superscript II reverse transcriptase and 0.3 µl RNase inhibitor (Promega Madison WI, USA) was incubated at 42°C for 90 min and denatured at 70°C for 15 min. The synthetized cDNA was tested by amplifying of GAPDH as a housekeeping gene using standard PCR conditions. For this, PCR was preformed using 2 µl of the tested cDNA as a template, 0.5 µl Taq polymerase (Sigma), 0.5 µl of dNTPs (10 mM), 0.5 µl of forward primer (5´-CTCGTCACTTGCAACTTGCTATTC-3´) and 0.5 µl of the reverse one (5´-CCAGGCATCCTTTAGACAGTCTTC-3´) at a concentration of 10 µM and 1x PCR buffer in a final reaction volume of 20 µl. Thermal cycler machine (My Cycler Thermal cycler, Bio-Rad Laboratories, CA, USA) was run for 1 cycle as a pre denaturation (5 min at 95°C), 40 cycles for amplification (denaturation: 1 min at 95°C, annealing: 30 sec at 60°C and extension: 30 sec at 72°C) followed by 10 min for

final extension at 72°C. Finally, products were visualized on a 2 % agarose gel using gel documentation system XR (Bio-Rad Laboratories, CA, USA).

3.2.3.3 Transcription level quantification

A total of 12 genes namely NRF2, KEAP1, NRF2 downstream antioxidant genes (SOD1, HMOX, PRDX1, NQO1, TXN and CAT) and genes involve in lipid metabolism (SREBP1, ACACA1, PPARAα and CPT2) were investigated for their expression using standard curve method as in the following steps:

Primer design

Sequence specific primers (Table 4) were designed for NRF2 mediated oxidative stress pathway related genes and selected lipid metabolism genes using primer3 online Software v.0.4.0 (http://frodo.wi.mit.edu/primer3/). All primers were obtained from Eurofins MWG synthesis GmbH (MWG Biotech, Eberberg, Germany).

Preparation of plasmid serial dilutions

Standard curves were generated using serial dilutions (consisting of 10^1-10^9 molecules) of each gene product including the endogenous control gene (GAPDH) to elucidate the amount of transcripts in unknown samples as following:

a. Gene specific PCR amplification and sequencing

PCR reaction was preformed for each primer set in final reaction volume of 20 µl containing 2 µl cDNA template, 0.5 µl of each specific primer (forward and reverse in a concentration of 10 pmol), 4 µl of 10x PCR buffer (Sigma-Aldrich), 0.5 µl of dNTP (10 mM), 0.5 µl of Taq polymerase (Sigma-Aldrich) and sterile purified water which was added up to 20 µl total reaction volume. The thermal cycler machine software was adjusted for amplification program of 40 cycles as described above.

Table 4: Details of specific primers designed for qPCR analysis in bovine embryos

Gene	Accession number	Primer sequences	Annealing temperature (°C)	Product size (bp)
NFE2L2	NM_001011678	F 5′-cccagtcttcactgctcctc-3′ R 5′-tcagccagcttgtcattttg-3′	55	165
KEAP1	NM_001101142.1	F 5′-tcaccagggaaggatctacg-3′ R 5′-agcggctcaacaggtacagt-3′	55	199
CAT	NM_001035386.1	F 5′-tgggacccaactatctccag-3′ R 5′-aagtgggtcctgtgttccag-3′	51	178
TXN1	NM_173968.3	F 5′-agctgccaagatggtgaaac-3′ R 5′-actctgcagcaacatcctga-3′	55	215
HMOX1	NM_001014912	F 5′-caaggagaaccccgtctaca-3′ R 5′-ccagacaggtctcccaggta-3′	55	225
PRDX1	NM_174431.1	F 5′-tggatcaacacacccaagaa-3′ R 5′-gtctcagcgtctcatccaca-3′	55	217
SOD1	NM_174615	F 5′-agaggcatgttggagacctg-3′ R 5′-cagcgttgccagtctttgta-3′	53	189
NQO1	NM_001034535.1	F 5′-aaccaacagaccagccaatc-3′ R 5′-cacagtgacctcccatcctt-3′	54	154
SREBP1	NM_001113302.1	F 5′-accgctcttccatcaatgac-3′ R 5′-ttcagcgatttgcttttgtg-3′	56	190
PPARAα	NM_001034036.1	F 5′-cctacgggaatggcttcata-3′ R 5′-gcacaataccctcctgcatt-3′	54	219
CPT2	NM_001045889.1	F 5′-cacaacatcctgtccaccag-3′ R 5′-ccttccaaggcatcaaacat-3′	54	209
ACACA1	NM_174224.2	F 5′-ctcttccgacaggttcaagc-3′ R 5′-accatcctggcaagtttcac-3′	55	248

After the end of the machine reaction program, the presence and specificity of the PCR product were detected by loading of 10 µl PCR product onto agarose gel with ethidium bromide, running in 1x TAE buffer using electrophoresis machine (BoRad, Munich, Germany). Finally, DNA bands were visualized and characterized using gel documentation system XR (Bio-Rad Laboratories, CA, USA).

Furthermore, the PCR products of the selected genes were sequenced to validate the identity of the genes. Sequencing reaction in final volume of 20 µl containing 5 µl PCR product, 2 µl reverse primer or forward primer (10 pmol), 4 µl master mix (DTCS) and 9 µl sterile purified water was performed for 30 cycles (at 96 ^{o}C for 20 s), followed by another one cycle (50 ^{o}C for 20 s) and a final cycle for 4 min at 60 ^{o}C. Consequently, 5 µl stop solution containing 1 µl glycogen, 2 µl 3M NAOAc (pH 5.2), 2 µl 0.1M EDTA (pH 8) was added to the sequencing reaction. The total reaction mix was then homogenized by vortexing and centrifuged after addition of 60 µl 100% ethanol for 15 min at 14000 rpm in 4 ^{o}C using refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany). After removing the supernatant, the pellets were washed twice in 70 % ethanol (200 µl), dried at room temperature and dissolved in 40 µl SLS. Next, the dissolved pellets were transferred to the sequencing plate and covered with mineral oil. CEQTM 8000 Genetic Analysis (Beckman Coulter, Krefeld, Germany) sequencing machine was used to identify and analysis the sample sequences. The similarity of the identified sequences and the original sequence was confirmed using the NCBI/BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

b. Genetic transformation and cloning

After confirmation of the designed primers specificity by comparing the sequenced PCR products of those genes with the original genes sequences, the sequenced PCR products were used to prepare a serial dilution for a standard curve. For this, QIAquick PCR Purification kit (Qiagen, Hilden, Germany) was used according to the manufacture instructions to purify the PCR product of each gene. Briefly, one volume of the PCR product was mixed with 5 volume of PB buffer, transferred to QIAquick spin column and centrifuged at room temperature for 1 min using Hermel Z200 M/H centrifuging machine (Hermle Labortechnik, Wehingen, Germany) at 13000 rpm. The PCR product was then washed again by adding 750 µl PE buffer to the column and centrifuged at room temperature for 1 min at 13000 rpm, followed by once more centrifugation at maximum speed for an additional 1 min. Finally, the purified PCR product was eluted in 30 µl elution buffer. Pure PCR fragments were then ligated to pGEM®-T easy vectors (Promega, WI, USA) by incubating 2 µl pure PCR product with 3 µl of ligation buffer, 0.5 µl pGEM®-T vector (50 ng / µl) and 0.5 µl DNA ligase enzyme (5 u/µl)

overnight at 4°C. The competent cells (E. coli "JM109" Strain) transformation was carried out in a sterile 15 ml falcon tube. In brief, the competent cells were removed from -80°C, thawed and kept in ice. About 4 µl of each ligation mixture were transferred to 15 ml sterilized falcon tube to which 60 µl of the competent cells was added and kept in ice. After gently flicking the tube, the transformation mixture was incubated in ice for 20 min followed by heat shock at 42°C in a water-bath for 1.5 min, then cold shock in ice for 2 min immediately after the heat shock. After that, 650-700 µl of Luria-Bertani (LB) broth was added to the mixture in the falcon tube and incubated at 37°C for 2 h at 110 rpm speed in shaking incubator (Incubated/Refrigerated Stackable Shaker Max Q 6000, Thermo scientific, IWA, USA). Before 20 min of the end of the incubation time, LB/ ampicillin/ IPTG/ X-gal culture plates were prepared under safety hood (HEAR safe Bio-flow safety hood, Heraeus instruments, Meckenheim, Germany). The surface of each LB agar/ampicillin plate for that was covered by 20 µl of 0.1 M IPTG and 20 µl of X-gal (50 mg/ml). At the end of bacterial incubation time, the transformed bacterial mixture was plated onto LB/ ampicillin/ IPTG/ X-gal plates in duplicate and incubated in the CO_2 incubator (Memmert CO_2 incubator, Fisher Scientific, Leicestershire, UK) at 37°C overnight. Colony plating assessment for successful DNA recombination was done by blue/white screening on LB agar/ampicillin/IPTG/X-gal plate. The successful cells transformed with recombinant plasmid will produce white colonies, since the successful insertion of the DNA fragments into pGEM®-T Easy vector interrupted the β-galactosidase coding sequence and led to change the blue color of cells transformed with non-recombinant plasmids to the white color. Four white colonies and two in blue color (to be used as a control) were picked from each plate and transferred to the 30 µl 1xPCR buffer. The same colonies were also inoculated to the 600 µl LB broth (continuing ampicillin) and incubated at 37°C in Incubated/Refrigerated Stackable Shaker Max Q 6000 (Thermo scientific, IWA, USA). In order to validate the transformation success and verify the insertion of the DNA fragments into pGEM®-T Easy vector, the 30 µl 1xPCR buffer including bacteria were heated for 15 min at 95°C in the thermal cycler (My Cycler Thermal cycler, Bio-Rad Laboratories, CA, USA). And out of the heated 30 µl 1xPCR buffer, 10 µl were used as a template to perform a PCR with specific primers (forward primer: 5′-TTGTAAACGCGGCCAGT-3′ and reverse primer: 5′-CAGGAAACAGCTATGACC-3′) of M13 promoter region of the vector. In addition to the 10 µl 1xPCR buffer, 0.5 µl

M13 forward primer, 0.5 µl M13 reverse primer, 0.5 µl dNTP (10 mM) and 0.5 U of Taq polymerase (Sigma) in 2 µl 10x PCR reaction buffer were mixed together in the PCR 0.2 ml tube. The PCR was run in the thermal cycler machine for pre denaturation at 95°C for 3 min, followed by 35 cycles for amplification (denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 1 min) and final extension for 10 min at 72°C. To evaluate the recombinant and non-recombinant DNA fragments of the white and blue colonies respectively, 10 µl of the M13 PCR products of the white and blue bacterial colonies were mixed with 2 µl loading buffer and loaded to 2% agarose gel stained with ethidium bromide. The DNA bands were then visualized under the gel documentation system XR (Bio-Rad Laboratories, CA, USA) and analyzed using Image Lab 2 software (Bio-Rad Laboratories, CA, USA). The incubated part of the transformed colonies in LB broth/ampicillin medium, which confirmed the presence of the recombinant plasmid containing the new DNA fragment, was then transferred to 15 ml sterile falcon tube. In order to increase the copy number of the recombinant plasmid, 5 ml LB broth/ampicillin medium were added to the bacterial suspension which was further cultured for 18-24 h at 37°C.

c. Recombinant plasmid extraction and purification

GenElute plasmid mini prep kit (Sigma-Aldreich, St.Lous, USA) was used to isolate and purify the recombinant plasmid from the transformed bacteria. According to the manufacturer's protocol, the bacterial cells were pelleted by centrifuging the bacterial suspension for 1 min at speed of 13000 rpm using Hermel Z200 M/H Centrifuge (Hermle, Wehingen, Germany). After removing the medium, 200 µl lysis buffer were added to the cell pellets and incubated at room temperature for 5 min. At the end of the incubation time, 350 µl neutralization/binding solution were added and the cell debris were precipitated by centrifuging the mixture for 10 min at maximum speed. Before the end of the centrifugation time, the GenElute Miniprep binding column was prepared by dropping of 500 µl preparation solution inside the binding column supplemented with microcentrifuge collection tube and centrifuging for 30 s at 13000 rpm. Afterward, the cleared lysate was transferred to the preprepared GenElute Miniprep binding column and centrifuged at 13000 rpm for 30 s. In order to purify the isolated plasmid in the binding column, 750 µl of DNA wash buffer diluted with ethanol was added to the

column followed by centrifugation for 30 s at 13000. After removing any flow-through liquid from the collection tube, the column was centrifuged again for 2 min at maximum speed to remove any chemical contaminants. Finally, the column was transferred into a new 1.7 ml collection tube and the plasmids were eluted in elution buffer. For this, 50 µl of the elution buffer were carefully dropped at the middle of the binding column membrane and centrifuged for 1 min at a speed of 13000 rpm. The plasmid concentration was determined using Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, DE, USA) and the specificity of gene cloning was further confirmed by M13 PCR product or plasmids sequencing using CEQ^{TM} 8000 sequencing machine

d. Plasmid serial dilutions preparation

Free online software, at http://molbiol.edu.ru/eng/scripts/h01_07.html, was used to convert the known DNA concentrations to known copy number of molecules. Briefly, the plasmid concentrations (ng/µl), which were obtained from Nanodrop 8000 spectrophotometer and the total size of the DNA including pGEM®-T easy vector and PCR fragment of each gene were submitted to the software. Based on the number of molecules obtained in 1 µl plasmid DNA, dilution that contains 10^9 molecules was prepared in 50 µl volume. Afterward, serial dilutions consisting of 10^8 to 10^1 copies of molecules were prepared from the dilution of 10^9 molecules.

Quantitative real time PCR

ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) instrument was used to quantify the transcript abundance of NRF2, NRF2 inhibitor KEAP1, NRF2 downstream antioxidant genes (SOD1, HMOX, PRDX1, NOQ1, TXN1 and CAT) and genes involved in lipid metabolism pathways such as SREBP1 and its downstream lipid biogenesis gene ACACA1, PPARAα and CPT2, which are involved in lipid catabolism process. Standard curves were generated using serial dilutions (consisting of 10^1-10^9 molecules) of each gene product, including the endogenous control gene (GAPDH), to elucidate the amount of transcript in unknown samples. The suitability of GAPDH as an endogenous control was validated by testing its expression stability among the experimental samples. Quantitative PCRs were

performed in 20 µl reaction volume containing 10 µl of SYBR green fluorescent detection dye master mix (Eppendorf, Hamburg, Germany), 2 µl of cDNA and an optimized amount of forward and reverse primers in ddH_2O to a volume of 8 µl. Following, initial denaturation step at 95 ^{o}C for 3 min, 40 cycles of 15 sec at 95 ^{o}C and 45 sec at 60 ^{o}C was used as thermal cycling program during real time PCR run. The relative standard curve method was used to determine the amount of transcripts in the experimental samples and the relative quantity is normalized using GAPDH as a housekeeping gene (Goossens et al. 2005) to compared transcript abundance differences between embryos cultured at 5% and 20% oxygen level at each of developmental stage.

3.2.4 Detection and localization of NRF2 and KEAP1 Proteins

Immunohistochemistry procedure (Ghanem et al. 2007, Goossens et al. 2011) was performed to detect and localize NRF2 and KEAP1 proteins in embryos from the treatment groups according to the experimental design of both experiments as described before. Briefly, 15 embryos from each excremental category were washed three times in in phosphate-buffered saline (PBS), fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBS. Fixed embryos were washed twice with glycine in PBS (GPBS), permeabilized with 0.5% (v/v) Triton-X100 (Sigma-Aldrich, Munich, Germany) in PBS for 4 hours at room temperature. The samples were then incubated in 3% normal donkey serum (Sigma-Aldrich, Munich, Germany) in PBS for 1 hour at room temperature, followed by incubation with specific primary antibodies against NRF2 (1:100 dilution, H300; Santa Cruz Biotechnology) or KEAP1 (1:100 dilution, H190; Santa Cruz Biotechnology) overnight at 4°C. Then, embryos were further incubated at room temperature for 1 hour in the dark with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat secondary antibody (1:100 dilution, Lifespan Biosciences). After that, a droplet of Vectashield mounting medium (Dabco; Acros, Geel, Belgium) containing 4',6-diamidino-2- phenylindole (DAPI) was used to stain the nuclei with blue color. Finally, samples were visualized under a CLSM LSM-780 confocal laser-scanning microscope (Carl Zeiss, Germany).

3.2.5 Intercellular ROS detection

In addition to blastocyst stage embryos from the two oxygen level in the first experiment, different competence categories of the 2-cell and blastocyst stage embryos in the second experiment were used to determine the ROS level using the H2DCFDA fluorescent probe (6-carboxy- 2',7'-dichlorodihydrofluorescin diacetate). Fifteen embryos from each group were incubated with 400µl of 5µM H2DCFDA (Invitrogen, Carlsbad, CA, USA) for 20 min at 37°C in dark. Samples were then washed twice in PBS and the images were captured immediately under Leica DM IRB inverted microscope (Leica, Bensheim, Germany) using green fluorescence filter.

3.2.6 Assessment of embryo mitochondrial activity

Mitochondrial activity of the embryos from experimental groups was determined using MitoTracker® Red CMXRos (Invitrogen, Carlsbad, CA, USA). For this, ten embryos from each group were incubated with 200 nM of MitoTracker red dye for 45 min followed by twice washing with PBS and were then fixed overnight at 4°C in 4% formaldehyde. Fixed specimens were mounted on the slide with vectashield (H-1200) containing DAPI. Images were acquired with the suitable wavelength for the dyes at 40x magnification under confocal laser scanning microscope, CLSM LSM-780 (Carl Zeiss, Germany).

3.2.7 Lipid droplets staining

Lipid droplets at blastocyst stage (first experiment) and 2-cell and blastocyst stage embryos (second experiment) from the experimental categories were determined using Oil red O staining according to established protocol (Ramirez-Zacarias et al. 1992) with slight modification. Briefly, the working solution was prepared by dissolving 4.2 g of Oil red O (Invitrogen, Carlsbad, CA, USA) in 1200 ml of absolute isopropanol, allowed settling overnight at room temperature. Oil red O working solution was then filtered, diluted with 900 ml of deionized water and stored at room temperature. Fifteen embryos from each category were fixed in 10% formalin for 60 min followed by twice washing using PBS. Thereafter, the embryos were incubated for 10 min in 60% isopropanol and

covered with the Oil red O solution for 60 min. After the Oil red O was removed, the embryos were rinsed 4-5 times in PBS. The stained embryos were then examined and the images were captured under inverted microscope, DM-IRB (Leica, Bensheim, Germany).

3.2.8 Assessment of blastocyst cell number

Total blastocyst cell number of the cultured embryos either under low (5%) or high oxygen level culture conditions was quantified using nuclear fluorescence staining with the glycerol-based Hoechst 33342 (Sigma-Aldrich, Munich, Germany) according to the manufacturer's procedure. Twenty blastocysts derived from 5% and 20% oxygen conditions were fixed for 5 min in a solution containing 2% formalin and 0.25% gluteraldehyde. The fixed blastocysts were mounted and stained for 10 min with glycerol based Hoechst 33342 (12.5 mg/ml) solution on clean glass slides. Stained nuclei appeared blue when visualized using an epi-fluorescent microscope (Olympus, Tokyo, Japan) fitted with a blue filter (excitation: 330-385 nm; emission: 420 nm; dichromatic: 400 nm). The cell number was scored for individual blastocysts from each culture group.

3.2.9 Cryotolerance test

The cryotolerance of bovine embryos was assessed as described previously (Li et al. 2006). Briefly, blastocysts at Day 7 and 8 from each experimental category were equilibrated in a solution of 10% ethylene glycol and 10% DMSO for 2 min. Samples were then exposed to vitrification solution containing 20% ethylene glycol and 20% DMSO. Blastocysts were loaded into an open-pulled straw, then immediately plunged into liquid nitrogen. After freezing, the embryos were thawed by immersing the embryo-end of the open pulled straw in 0.3 mol/L sucrose for 5 min, then in 0.2 mol/L sucrose for 5 min and then in holding medium for 5 min. Finally, the embryos were cultured to determine hatching rate after thawing.

3.2.10 Statistical Analysis

Data were statistically analyzed using Statistical Analysis System (SAS) version 9.1 (SAS Institute Inc., Cary, NC, USA). Mean developmental and hatching rates of different treatment groups of embryos were analyzed by one-way ANOVA followed by a multiple pairwise comparison (Tukey test). SAS General Linear Model (GLM) was used to analyze the relative mRNA expression data. Differences in mean values were tested among treatments using ANOVA followed by a multiple pairwise comparison using t-test. Differences were considered significant ($P<0.05$).

4 Results

4.1 In vitro development of bovine embryos under 5% and 20% oxygen levels

Embryo developmental competence was evaluated in terms of cleavage and blastocyst rates during in vitro culture under high or low O_2 levels. The cleavage rate did not differ significantly between groups, namely 79.1% for 20% and 77.8% for 5% oxygen conditions (Table 5). On the other hand, culturing embryos under 5% oxygen resulted in significantly higher ($P < 0.05$) blastocyst rates than those cultured at 20% oxygen (37.8% vs. 25.4%, respectively).

Table 5: Developmental rates of bovine zygotes cultured under high or low oxygen level culture conditions until blastocyst stage.

Oxygen level	No. of zygotes	Cleavage rate		Blastocyst rate			
				Day 7		Day 8	
		n	(%)	n	(%)	n	(%)
5 % O_2	1372	1085	79.1	400	29.2 [a]	118	8.6
20 % O_2	1381	1074	77.8	224	16.2 [b]	127	9.2

[ab] Values with different superscripts within columns differ significantly.

4.2 Expression analysis of NRF2 mediated oxidative stress response and lipid metabolism pathway genes in bovine embryos cultured in vitro under oxidative stress conditions

4.2.1 High oxygen level induced oxidative stress under in vitro culture conditions

In order to validate the effect of the oxygen level as a source of oxidative stress in the standard in vitro culture system, we have compared ROS level in the embryos derived from high (20%) and low (5%) oxygen culture conditions. Subsequently, higher level of ROS have been detected in embryos cultured under high (20%) oxygen level after staining with ROS detector (H2DCFDA) compared to those cultured under 5% oxygen level (Figure 13).

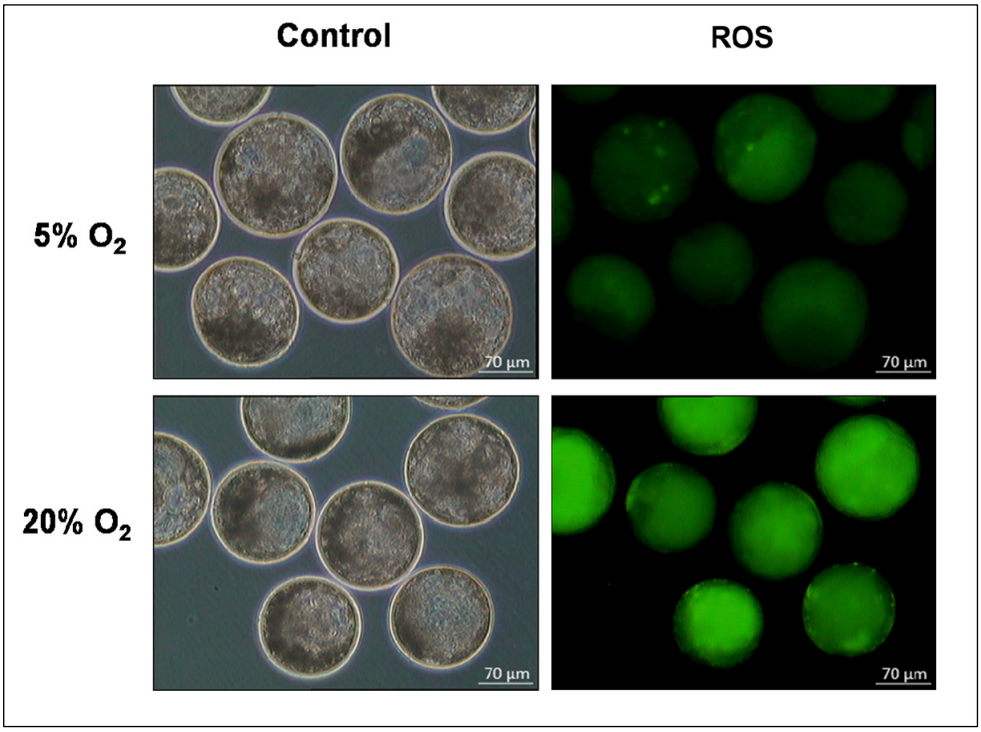


Figure 13: ROS level of bovine blastocyst embryos derived from 5% and 20% oxygen culture environments. Scale bars, 70 µm.

4.2.2 Expression analysis of NRF2 mediated oxidative stress response and lipid metabolism pathway genes in bovine embryos cultured at 5% and 20% oxygen

In order to quantify the mRNA expression level of the selected genes in the embryos cultured under high (20%) or low (5%) oxygen level culture conditions using qPCR, the suitability of GAPDH, as an endogenous control, under the experimental conditions was confirmed by analyzing its expression in the experimental groups of embryos. Under both oxygen level culture conditions, the differences in GAPDH transcription level did not reach to the statistical significance limits (Figure 14)

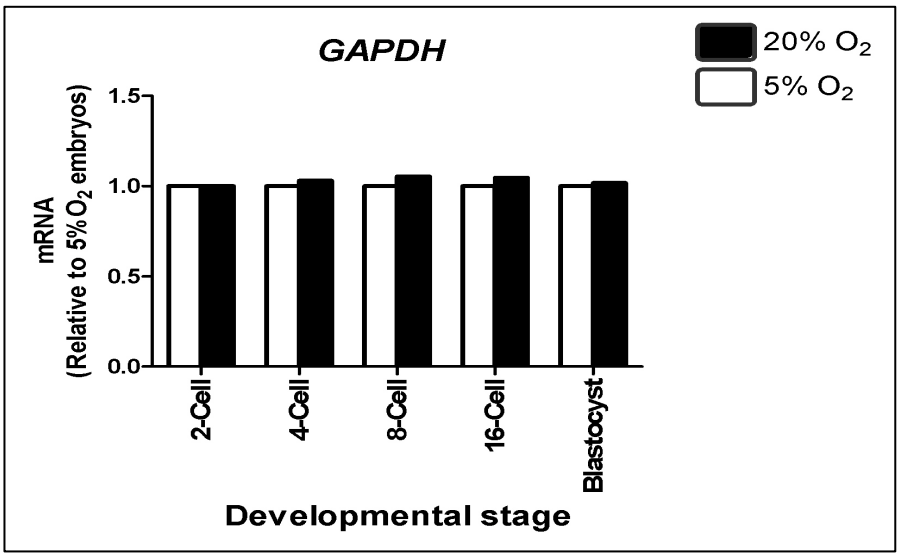


Figure 14: mRNA transcript abundance of housekeeping gene (GAPDH) in early developmental stages of bovine embryos cultured in vitro under 5 or 20% oxygen atmosphere. No statistically significant differences were observed ($P < 0.05$).

4.2.2.1 Oxidative stress activated the embryonic NRF2 in a stage-specific manner

To investigate the activity of NRF2 mediated oxidative stress response of embryos due to elevated ROS under oxidative stress conditions, gene expression level of NRF2 and its inhibitor (KEAP1) during different stages of embryonic development was compared under 5% (low) and 20% (high) oxygen levels. Results showed that, the relative abundance of NRF2 transcript was significantly higher in embryos cultured at high (20%) oxygen level than that of low oxygen group. This was mainly evident at 8-cell, 16-cell and blastocyst stage embryos. However, at 2-cell and 4-cell stages, there were no significant differences between the groups. An opposite trend of expression was evident in all stages of development for NRF2 inhibitor KEAP1 gene. The mRNA expression level of KEAP1 was up-regulated in 8-cell, 16-cell and blastocyst stages in embryo under low (5%) compared to high (20%) oxygen level (Figure 15).

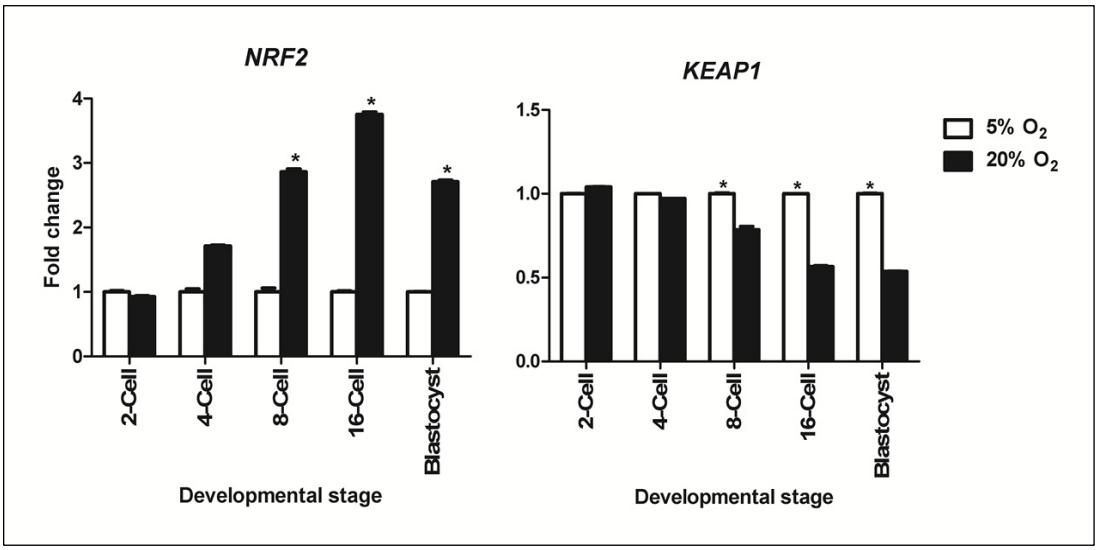


Figure 15: Expression levels of NRF2 and its inhibitor KEAP1 at various developmental stages. Expression was compared between in vitro-produced bovine embryos cultured under 5% (white bars) and 20% (black bars) oxygen. * P< 0.05.

Immunofluorescence staining for NRF2 and KEAP1 proteins at blastocyst stage embryos from 20% versus 5% oxygen conditions paralleled the transcript abundance. Higher florescence intensity of nuclear NRF2 protein was detected in blastocysts from the 20% oxygen culture compared to the 5% oxygen condition. In contrast, blastocysts from 20% oxygen conditions expressed less KEAP1 protein (Figure 16).

4.2.2.2 Oxidative stress induced changes in the expression of NRF2-regulated antioxidant genes

To confirm whether the activation of NRF2 is associated with its downstream antioxidant genes, expression of NRF2 antioxidant downstream genes, namely SOD, CAT, PRDX1, HMOX1, NQO1 and TXN1, was investigated in pre-implantation stage embryos derived from 20% or 5% oxygen environments. Interestingly, the expression of SOD1, CAT and PRDX1 genes was higher in young, cleavage-stage embryos cultured in 5% than in 20% oxygen until the 8-cell stage, when the expression pattern reversed. Higher expression levels of NQO1, TXN1 and HMOX1 were detected at the 8- and 16-cell stages for embryos cultured at 20% compared to 5% oxygen level (Figure 17).

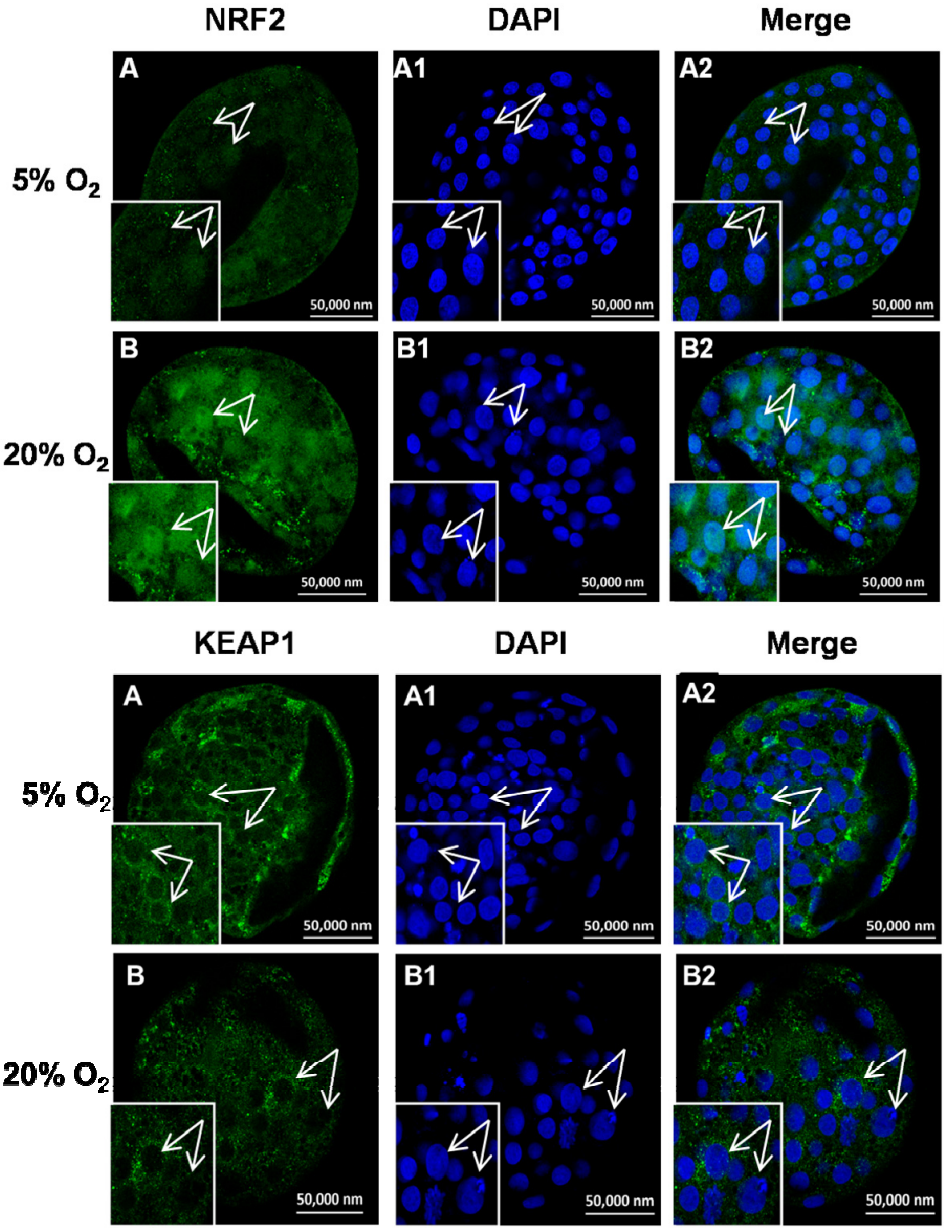


Figure 16: Protein expression of NRF2 and KEAP1 in bovine blastocyst stage embryos cultured under 5% or 20% oxygen conditions. Labels A&B showing green fluorescence signals reveal NRF2 and KEAP1 protein localization, while blue (A1&B1) shows nuclear staining using DAPI. A2&B2 are merged images. Original magnification 40x. Scale bars, 50 μm.

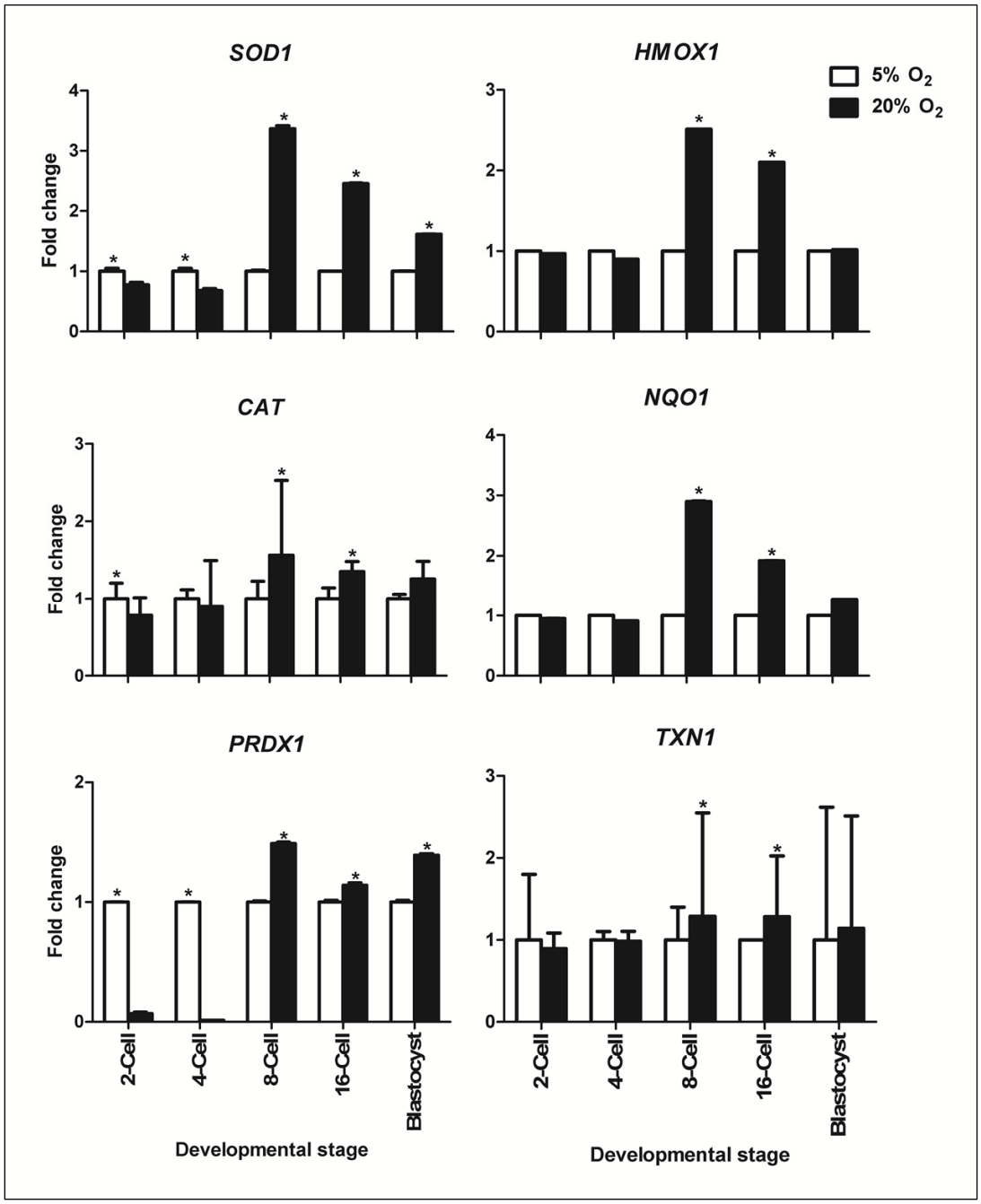


Figure 17: Expression levels of NRF2 downstream antioxidant regulated genes (SOD1, CAT1, PRDX1, HMOX1, NQO1 and TXN1) at various developmental stages. Expression was compared between in vitro-produced bovine embryos cultured under 5% (white bars) and 20% (black bars) oxygen. * $P < 0.05$.

4.2.2.3 Oxidative stress induced changes in the expression of lipid metabolism genes

In addition to NRF2 mediated oxidative stress pathway related genes, the present study also looked at the effect of oxidative stress on the lipogenic pathway, specifically evaluating the expression of the lipid metabolism genes (SREBP1, ACACA1, CPT2 and PPARAα) in the same groups of embryos. Overall, the expression of SREBP1, ACACA1, CPT2 and PPARAα genes were higher in embryos derived from the 20% oxygen condition compared to those from 5% oxygen at various stages of development (Figure 18).

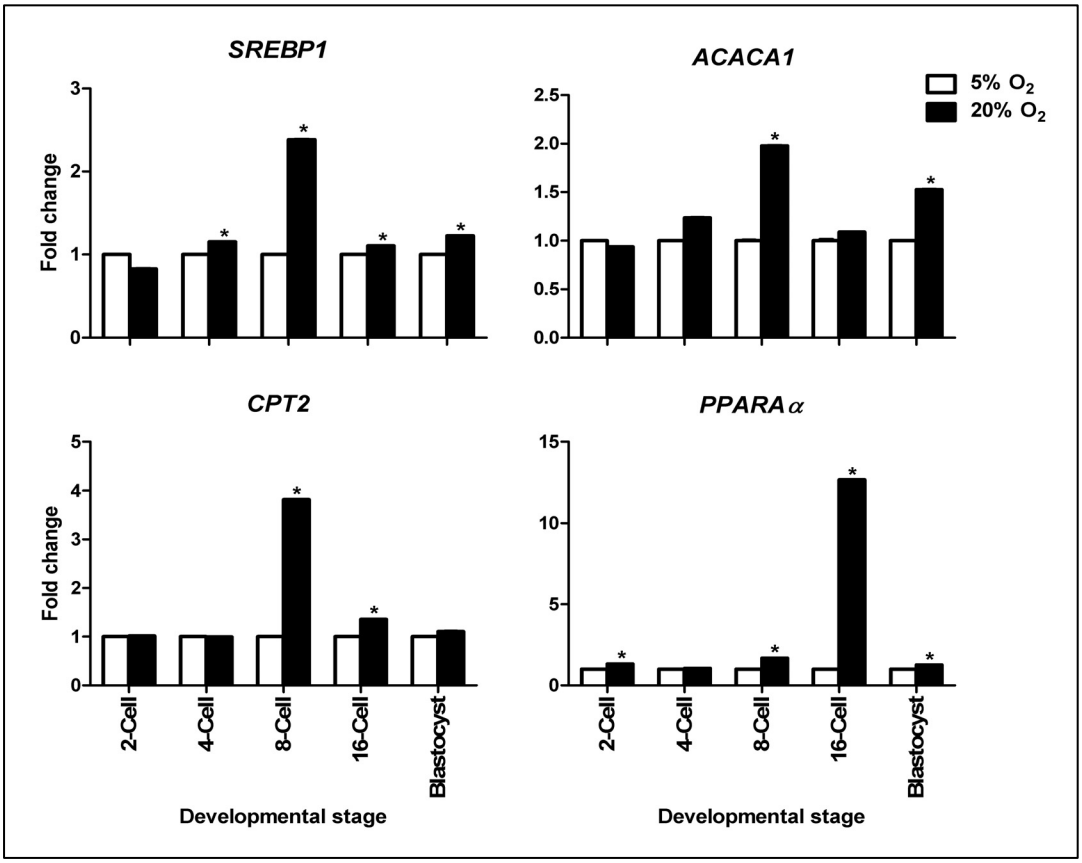


Figure 18: The expression pattern of selected lipid metabolism genes (SREBP1, ACACA1, CPT2 and PPARAα) at various developmental stages. Expression was compared between in vitro produced bovine embryos cultured under 5% (white bars) and 20% (black bars) oxygen. * P< 0.05.

At 4-, 8-, 16-cell and blastocyst stages of development, SREBP1 transcript was significantly higher in embryos cultured at high (20%) oxygen level than that of low oxygen group. A similar trend of expression was evident in 8-cell and blastocyst stage embryos for SREBP1 downstream lipid biogenesis gene ACACA1. Higher expression levels of lipid catabolism gene CPT2 was observed at the time of major genomic activation, which is at 8- to 16-cell stage in bovine, till blastocyst stage of development under high compared to low oxygen level treatment group. In addition, except 4-cell stage, PPARAα transcript was significantly higher in earlier stages of development.

4.2.3 Oxidative stress reduced the mitochondrial activity of the bovine embryos

In order to evaluate the effect of elevated ROS on the mitochondria of the bovine embryos, mitochondrial activity was assessed in blastocysts derived from low (5%) and high (20%) oxygen tension culture conditions.

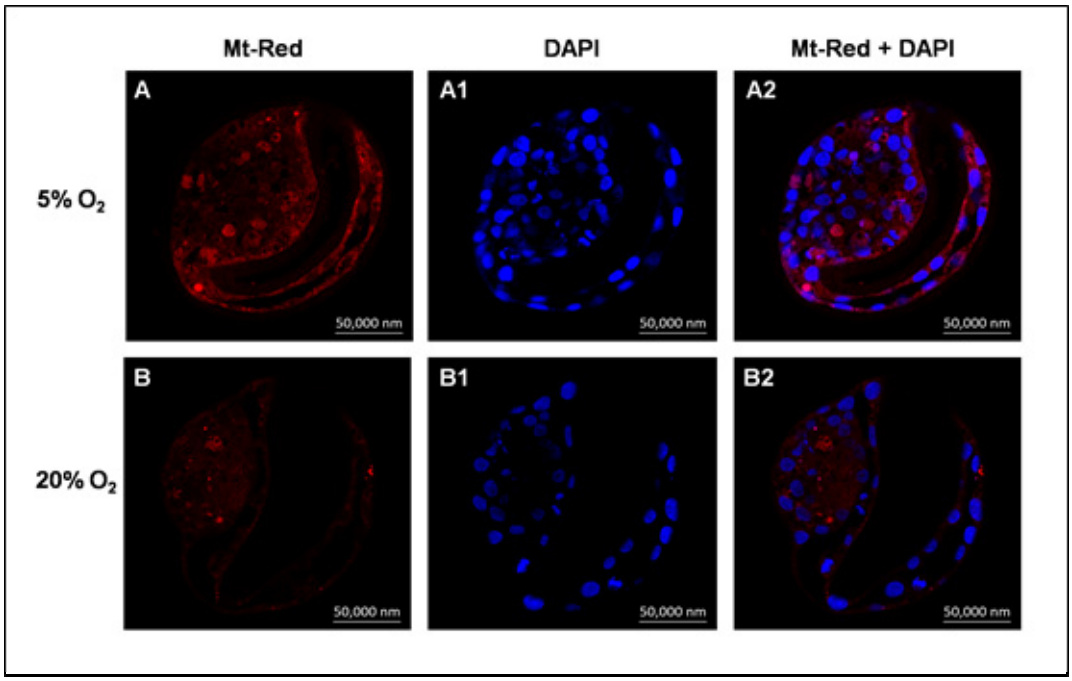


Figure 19: Mitochondrial activity of bovine blastocysts derived from 5% and 20% oxygen conditions. Mitochondria were stained with MitoTracker red (A & B) and nuclei were stained with DAPI (A1 & B1). A2 and B2 show merged pictures of the protein and nuclei. Original magnification 40x. Scale bars, 50 µm.

As shown in Figure 19, blastocysts cultured in 5% oxygen exhibited higher mitochondrial activity compared to blastocysts derived from 20% oxygen culture, as evidenced by the high intensity of florescence dye detected by confocal microscopy.

4.2.4 Effect of oxidative stress on embryo lipid accumulation

In order to know whether the embryo metabolism, in terms of accumulation of lipid, was affected by the increased oxidative stress under in vitro culture conditions, Oil Red O staining was performed to detect the intracellular lipid content of blastocysts derived from 5% and 20% oxygen level. Indeed, culturing the embryos under high oxygen tension (20% O_2) lead to a clear increase in lipid accumulation at blastocyst stage compared to low (5% O_2) oxygen tension (Figure 20).

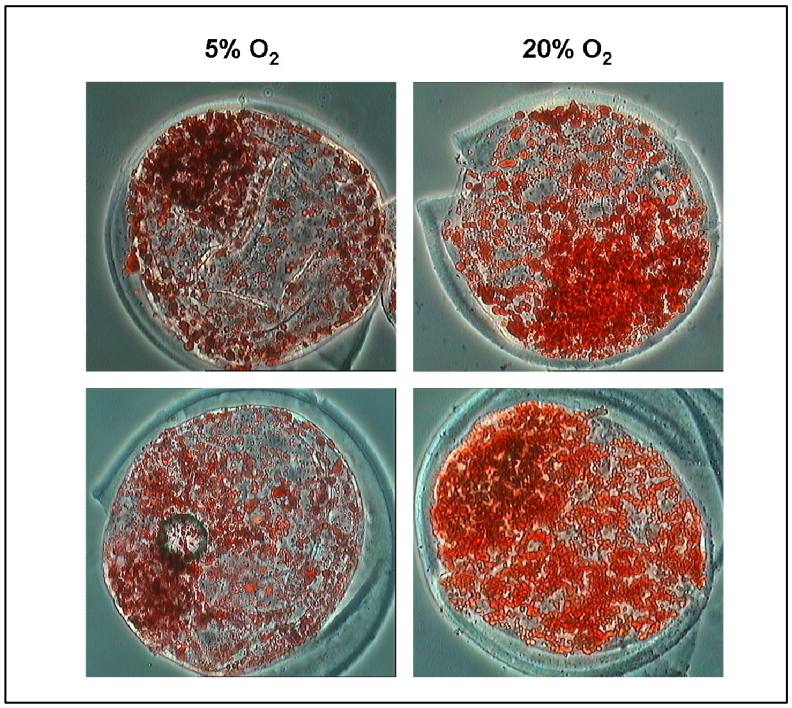


Figure 20: Lipid droplet accumulation as stained by Oil Red O stain in blastocysts generated under low (5%) and high (20%) oxygen culture environment. Original magnification 40x. Scale bars, 70 µm.

4.2.5 Blastocyst cell number according to environmental oxygen tension

Blastocyst cell number were counted in two group of embryos each containing 20 embryos derived from high or low oxygen tension culture conditions. Blastocysts derived from 20% oxygen had significantly fewer cells (132 ± 29.5) compared to those from the 5% oxygen condition (155 ± 20).

4.3 Association of NRF2 mediated oxidative stress response pathway activity with the developmental competence of bovine in vitro produced embryos

4.3.1 NRF2 and KEAP1 gene expression in relation to embryo competence

To investigate whether the activity of NRF2 mediated oxidative stress pathway in pre-implantation bovine embryo under oxidative stress conditions is associated with embryo competence, cleavage and blastocyst developmental rate was taken as a model under high and low oxygen levels. The time of the first cleavage and time to reach to blastocyst stage were considered to categorize embryos as competent (early 2-cell and early blastocyst) and non-competent (late 2-cell and late blastocyst). Accordingly, the expression levels of NRF2 and KEAP1 were investigated in competent vs. non competent embryos either at 2-cell or blastocyst stage embryos. No significant differences were observed in the expression of NRF2 and its inhibitor (KEAP1) genes between early and late cleaving 2-cell stage embryos under low oxygen level. However both NRF2 and KEAP1 transcripts were higher in the late cleaving 2-cell stage compared to early ones under 20% oxygen tension (Figure 21). On the other hand, NRF2 mRNA was higher in early developing blastocyst embryos compared to the late developing ones in both 5% and 20% oxygen culture conditions. Moreover, a reverse expression trend was found for its inhibitor, KEAP1 (Figure 22).

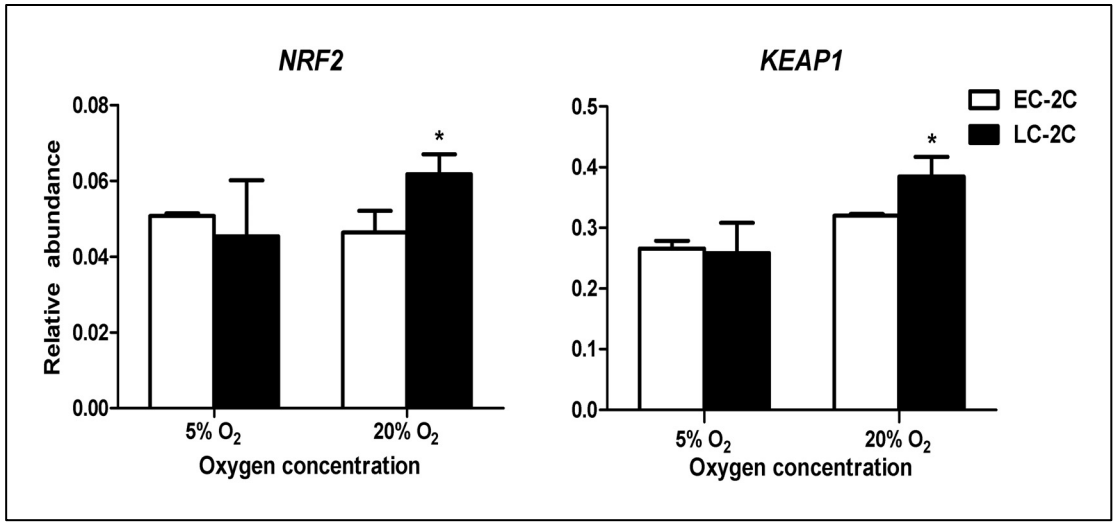


Figure 21: Relative abundance of NRF2 and KEAP1 genes in early competent cleaving 2-cell stage embryos (EC-2C, white bars) and late non-competent cleaving 2-cell (LC-2C, black bars) derived from 5% or 20% oxygen level. * P< 0.05.

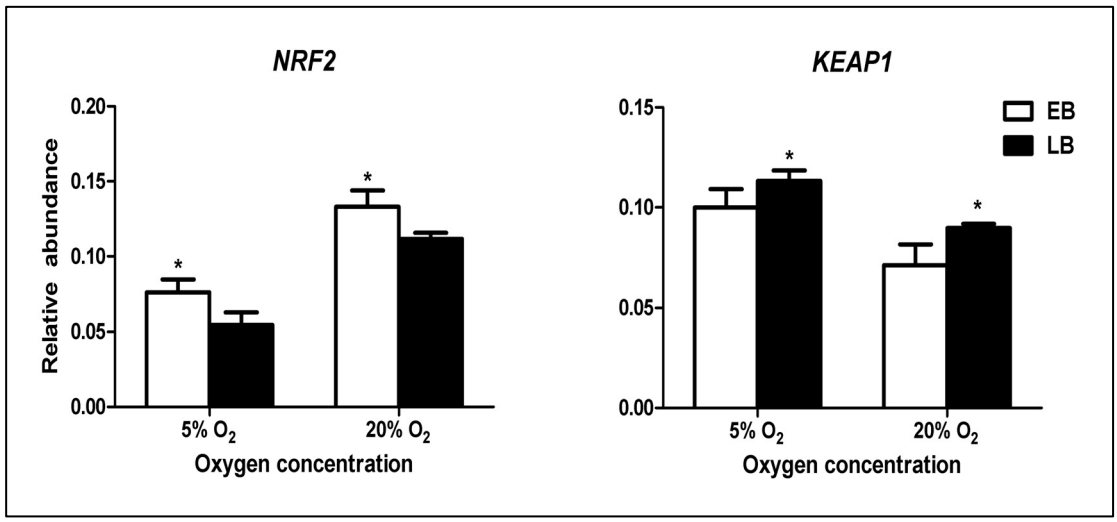


Figure 22: Relative abundance of NRF2 and KEAP1 genes in early competent blastocysts (EB, white bars) and late non-competent blastocysts (LB, black bars) derived from low (5%) or high (20%) oxygen level culture conditions. * P< 0.05.

4.3.2 NRF2 and KEAP1 protein expression in relation to embryo competence

Immunohistochemestry analysis was conducted to evaluate the expression level and localization of NRF2 and KEAP1 proteins in competent vs. non competent embryos generated under low (5%) and high (20%) atmospheric oxygen levels. As shown in Figure 23, 24 and 25, the protein expression level was in accordance with NRF2 and KEAP1 mRNA levels either in competent or in non-competent embryos derived from 5% (low) or 20% (high) oxygen tension culture conditions. In the in vitro generated 2-cell stage embryos, NRF2 and KEAP1 proteins showed the same immunohistochemical staining pattern (Figure 23).

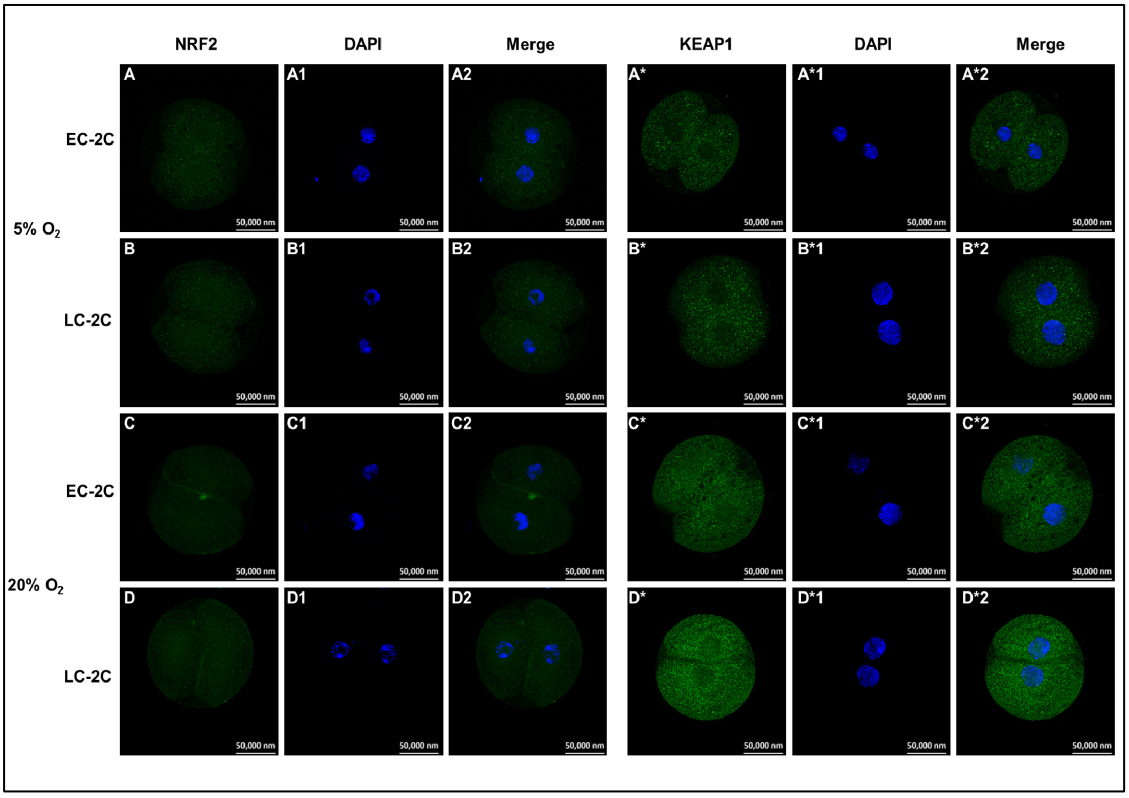


Figure 23: Expression and localization of NRF2 and KEAP1 proteins in early competent (EC-2C) vs. late in competent (LC-2C) cleaving 2-cell stage bovine embryos generated under 5% or 20% oxygen conditions. Labels A-D & A*-D* show NRF2 and KEAP1 proteins respectively in green, while blue (A1-D1& A*1-D*1) showed nuclear staining using DAPI. A2-D2& A*2-D*2 are merged images. Original magnification 40x. Scale bars, 50 µm.

No clear differences were detected for the expression of NRF2 and KEAP1 proteins between early vs. late cleaving 2-cell embryos under low oxygen tension. Conversely, the expression of both proteins was slightly higher in late cleaving 2-cell embryos under high oxygen tension culture condition.

More KEAP1 protein was detected in cytoplasm compared to the nucleus of the early and late 2-cell embryos cultured either under high or low oxygen levels, while NRF2 was detected equally in both cytoplasm and nucleus.

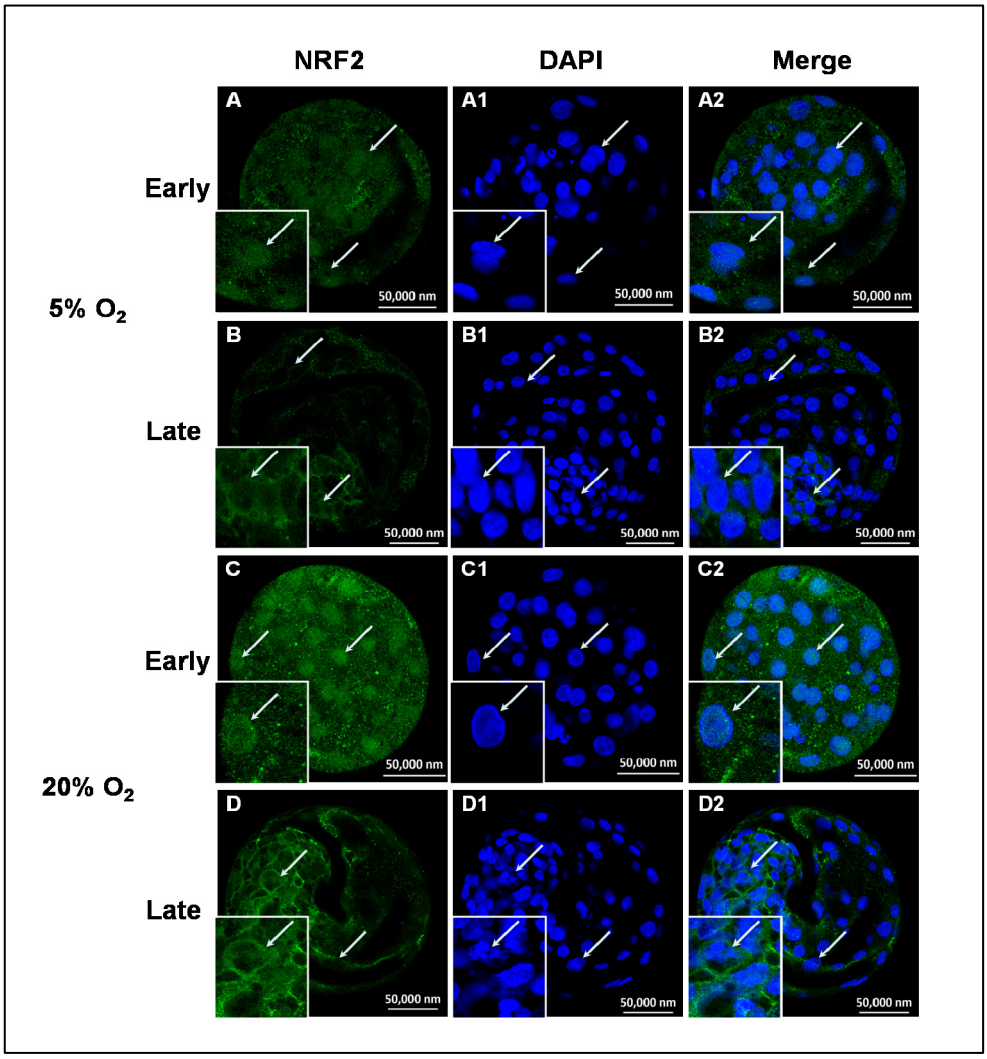


Figure 24: Localization of NRF2 protein in early (competent) (A & C) versus late (non-competent) (B & D) bovine blastocyst-stage embryos cultured under 5% or 20% oxygen conditions. Labels A, B, C & D show NRF2 protein in green, while A1, B1, C1 & D1 show nuclear staining in blue (DAPI). A2, B2, C2 & D2 are merged images of the protein and nuclei staining. Original magnification 40x. Scale bars, 50 µm.

At blastocyst stage of development, higher NRF2 protein was detected in both competent and non-competent blastocysts derived from 20% compared to 5% oxygen conditions (Figure 24). Conversely, the protein expression analyses of KEAP1 revealed lower level of KEAP1 in high oxygen tension (20% O_2) embryo group compared to low oxygen tension (5% O_2) group (Figure 25).

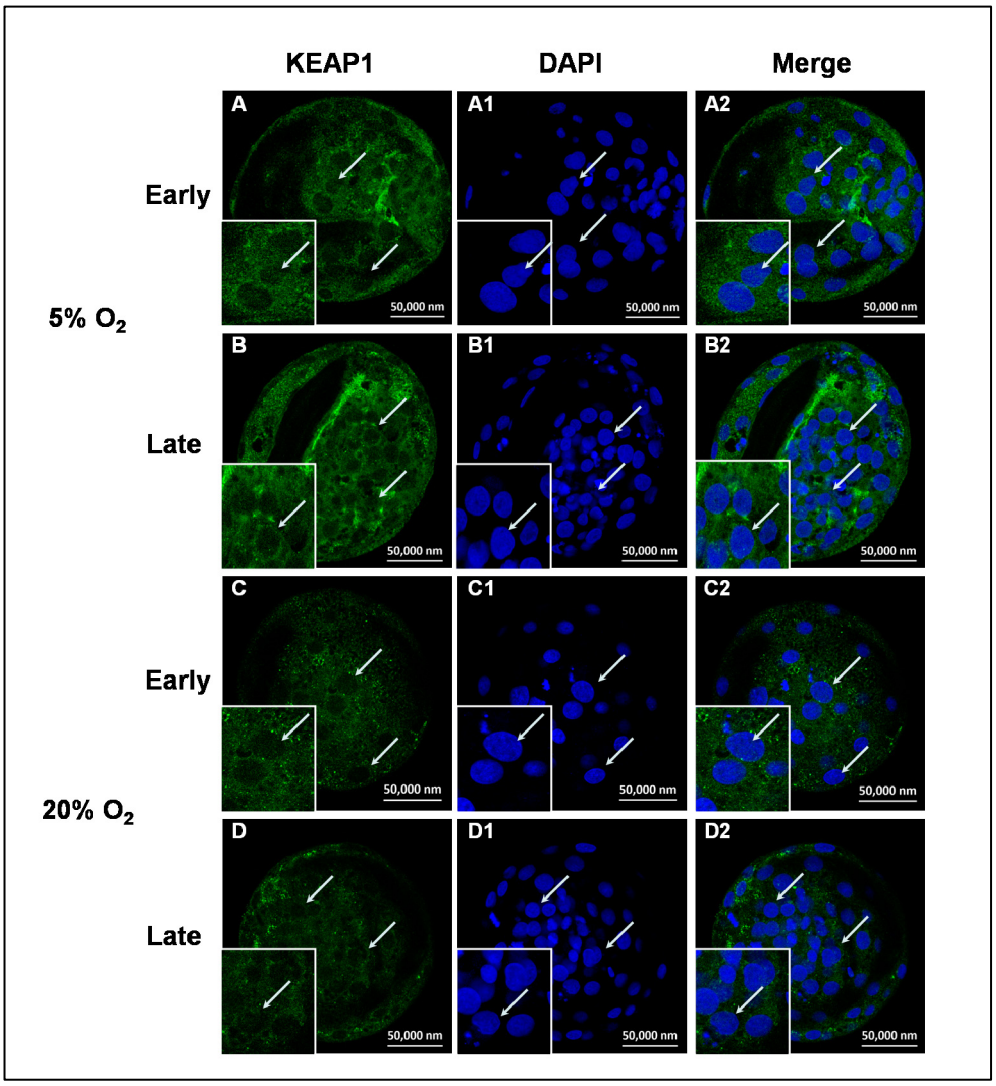


Figure 25: Localization of KEAP1 protein in early (competent) (A & C) versus late (non-competent) (B & D) bovine blastocyst stage embryos cultured under 5% or 20% oxygen conditions. Labels A, B, C & D show KEAP1 protein in green, while A1, B1, C1 & D1 show nuclear staining in blue (DAPI). A2, B2, C2 & D2 are merged images of the protein and nuclei staining. Original magnification 40x. Scale bars, 50 µm.

Moreover, under the same oxidative stress level, the transcription activity of NRF2 was higher in early blastocysts with clear accumulation of the protein in the nuclei of these embryos. In contrary, the late blastocyst embryos showed more cytoplasmic NRF2 compared to nuclear protein, showing less activity of NRF2, in those embryos (Figure 24). In addition, more KEAP1 protein signal could be observed in embryo cytoplasm than in the nuclei of both early and late blastocyst stage embryos either under 5% or 20% oxygen level culture conditions (Figure 25).

4.3.3 Expression of NRF2 downstream antioxidant genes in competent versus non-competent embryos

Four NRF2 antioxidant regulated genes (PRDX1, SOD1, CAT1 and TXN1) were selected for gene expression analysis in the early competent vs late incompetent embryos derived from high and low oxygen tension culture conditions. Results revealed that both PRDX1 and SOD1 antioxidants showed significant differences under high (20%) oxygen level in late compared to early cleaving 2-cell stage embryos (Figure 26). In contrast, CAT and TXN1 did not show any significant differences at the same oxygen level. Similarly except SOD1 gene, which was significantly higher at late cleaving 2-cell stage embryos, all NRF2 downstream antioxidant genes (PRDX1, CAT1 and TXN1) were not different between early and late cleaving 2-cell embryos under low oxygen tension (Figure 26).

At blastocyst stage, generally, the gene expression pattern results supported the findings of the first experiment, where the expression level of NRF2 downstream antioxidant related genes (PRDX1, SOD1, CAT1 and TXN1) was high in the 20% oxygen group (Figure 27). Moreover, consistent with the expression of NRF2, higher expression of PRDX1, SOD1, CAT1 and TXN1 antioxidants was found in competent blastocysts compared to their non-competent counterparts at both oxygen levels (Figure 27).

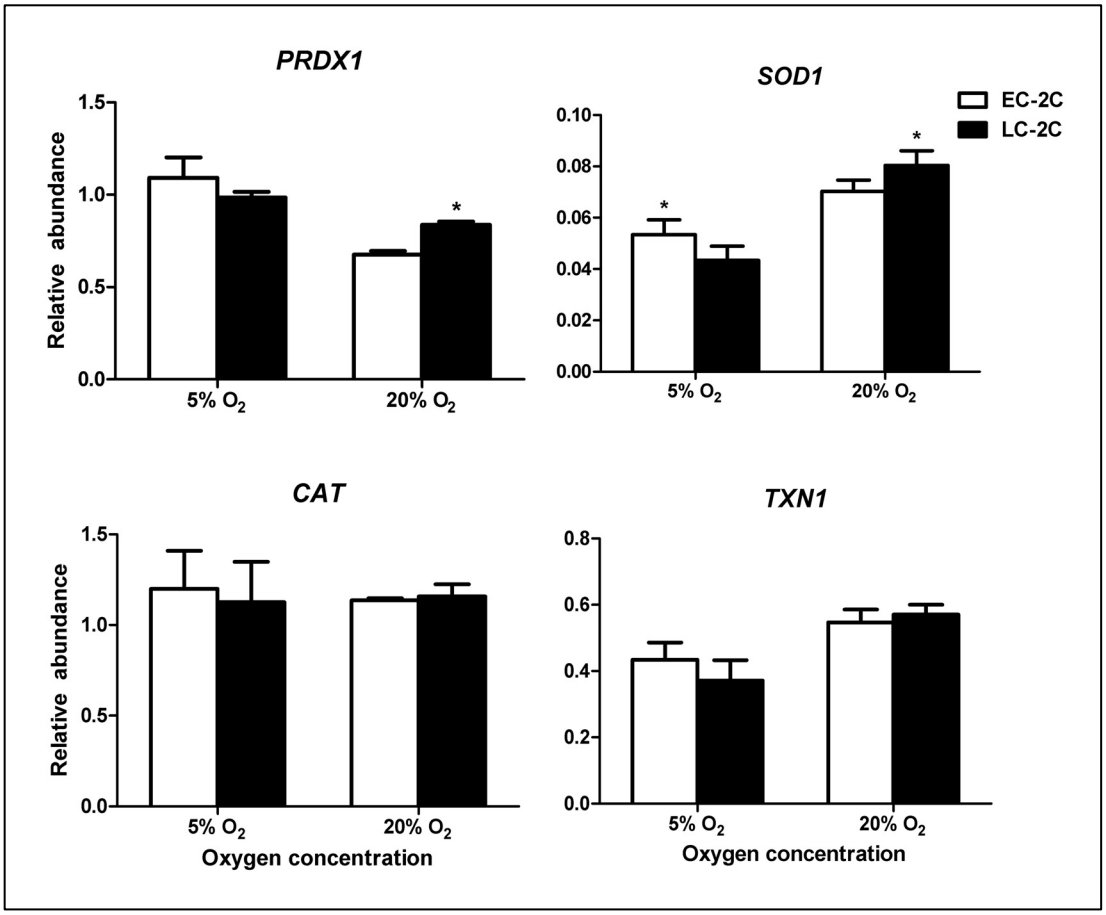


Figure 26: Relative abundance of NRF2 antioxidant downstream regulated genes (PRDX1, SOD1, CAT1 and TXN1) in early cleaving 2-cell (EC-2C, white bars) vs. late cleaving 2-cell (LC-2C, black bars) derived from low (5%) or high (20%) oxygen level culture conditions. * P< 0.05.

4.3.4 Expression of genes related to lipid metabolism genes in competent vs. non-competent embryos

Two lipid metabolism genes namely SREBP1, which is involved in lipid biosynthesis, and CPT2, which plays a central role in lipid beta-oxidation in mitochondria, were selected for gene expression analysis using qPCR. Although the gene expression analysis results of SREBP1 didn't show any significant differences between early and late cleaving 2-cell stage embryos under high or low oxygen level, its expression was increased as a result of elevated oxygen level under in vitro culture atmosphere (Figure 28).

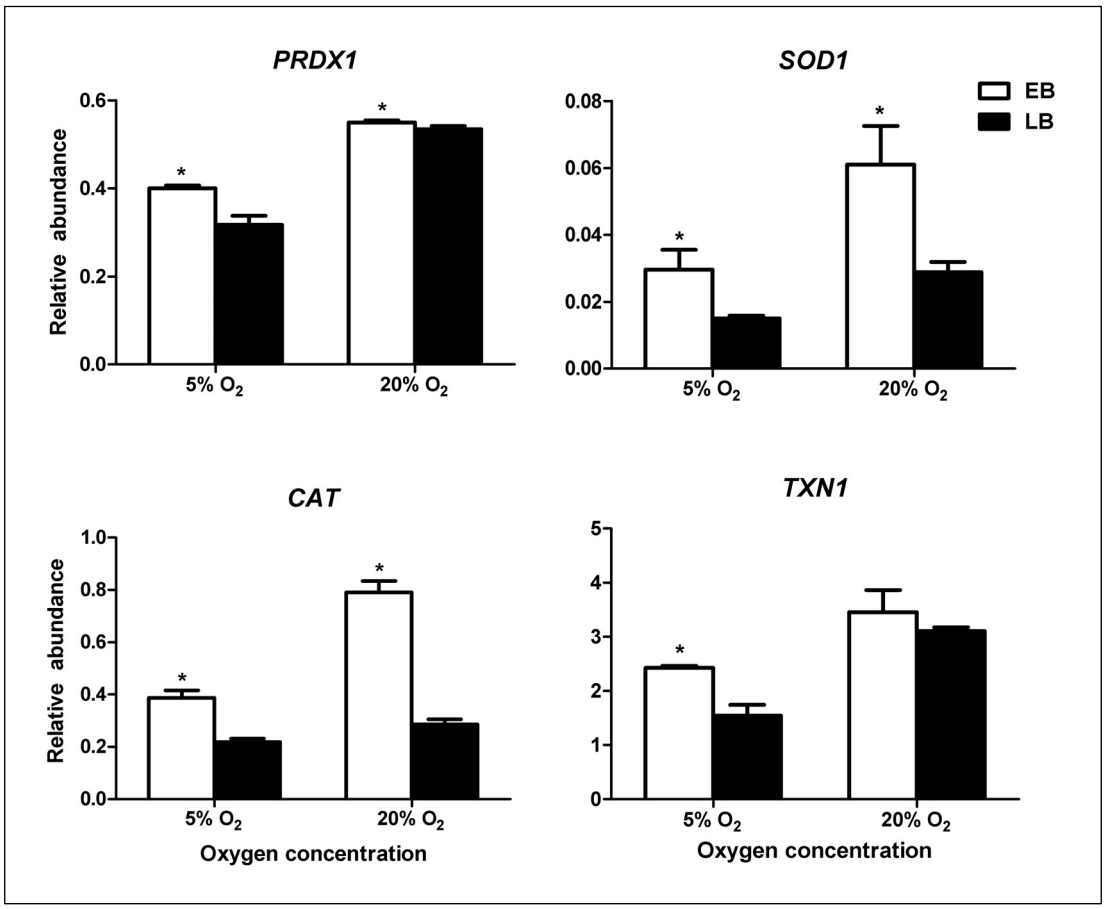


Figure 27: Relative abundance of NRF2 antioxidant downstream regulated genes (PRDX1, SOD1, CAT1 and TXN1) in early blastocyst (EB, white bars) vs. late blastocyst (LB, black bars) derived from low (5%) or high (20%) oxygen level culture conditions. * $P < 0.05$.

The CPT2 gene mRNA level was found to be higher ($P \leq 0.05$) in early cleaving 2-cell embryos at 5% oxygen level, while no difference was observed under 20% oxygen level (Figure 28). In addition, at blastocyst stage, while the expression of SREBP1 gene was significantly different between early vs. late blastocyst stage only at 5% oxygen concentration, CPT2 mRNA expression was significantly higher in early blastocyst compared to late ones at both oxygen levels (Figure 28).

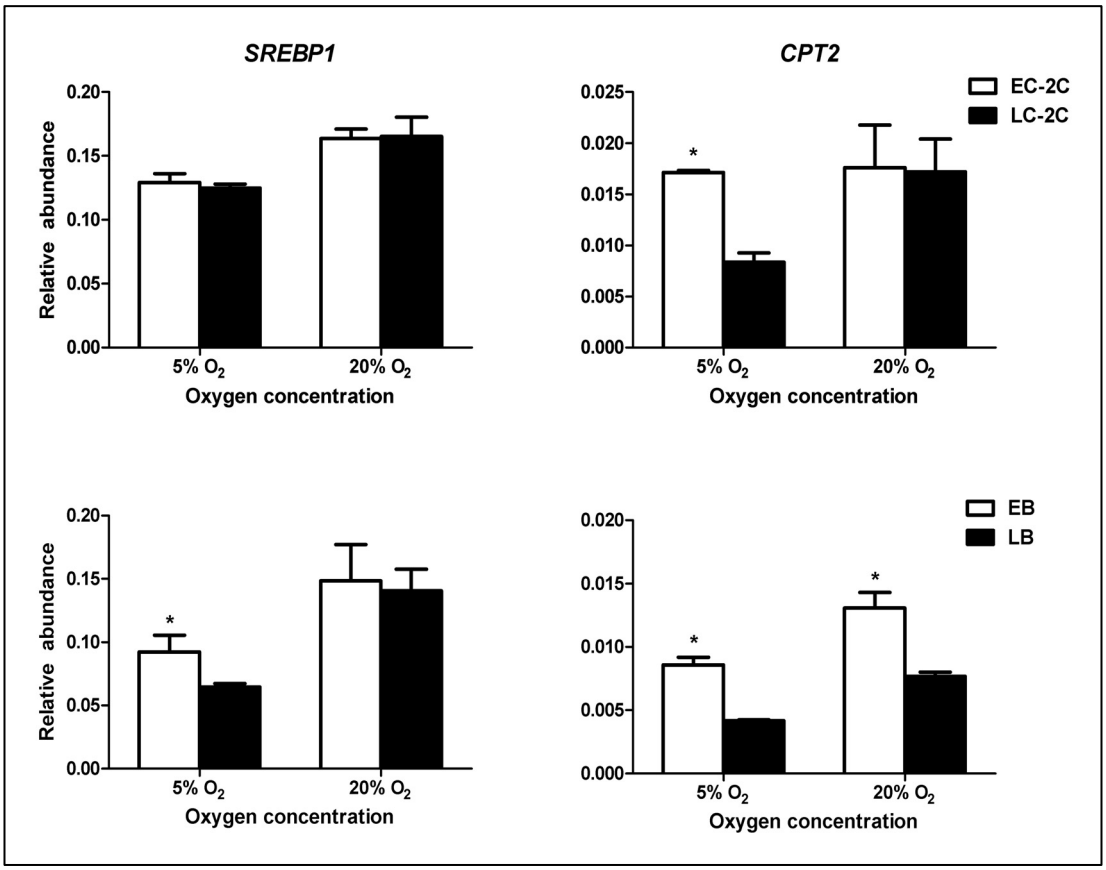


Figure 28: Relative abundance of lipid metabolism related genes (SREBP1 and CPT2) in early cleaving 2-cell (EC-2C, white bars) vs. late cleaving 2-cell (LC-2C, black bars), and early blastocyst (EB, white bars) vs. late blastocyst (LB, black bars) derived from low (5%) or high (20%) oxygen level culture conditions. * P< 0.05.

4.3.5 ROS level in relation to embryo competence under low and high oxygen tension

In order to investigate whether the different activity levels of NRF2 in different competent group embryos at both mRNA and protein level is associated with the ROS level, the intercellular ROS level were investigated using green fluorescent probe (H2DCFDA) in early and late generated embryos under high and low oxygen tension culture conditions. At both oxygen levels, intracellular ROS of both early cleaving 2-cell and blastocyst stage embryos were lower than their late developing embryo counterparts (Figure 29).

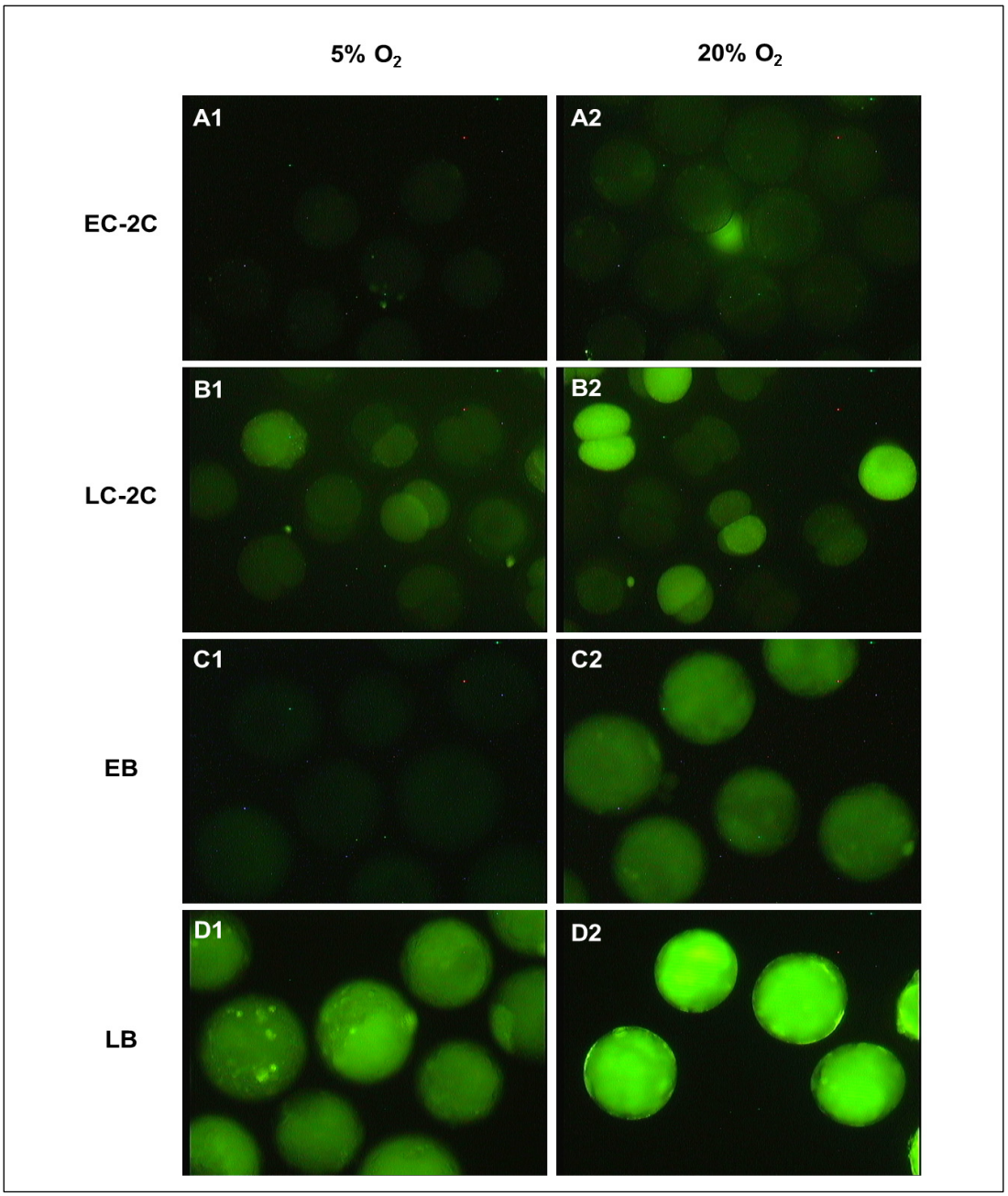


Figure 29: ROS staining of early cleaving 2-cell (EC-2C) vs. late cleaving 2-cell (LC-2C), and early blastocysts (EB) vs. late blastocysts (LB) derived form 5% and 20% oxygen conditions. Labels A1-D1 show ROS (green) in all competent categories under low (5%) oxygen level, while A2-D2 show ROS in all categories of embryos under high (20%) oxygen level culture conditions. Original magnification 40x. Scale bars, 70 µm.

4.3.6 Mitochondrial activity alterations

To investigate the effect of ROS accumulation levels on the mitochondrial activity in the early and late embryos, which have shown to have varying activity of NRF2, the activity of the mitochondria were determined at 2-cell and blastocyst stages either under high or low oxygen tension conditions. Results demonstrated that, early 2-cell and blastocyst stage embryos which were considered developmentally competent have more mitochondrial activity than late 2-cell and blastocyst counterparts (Figure 30 and 31 respectively).

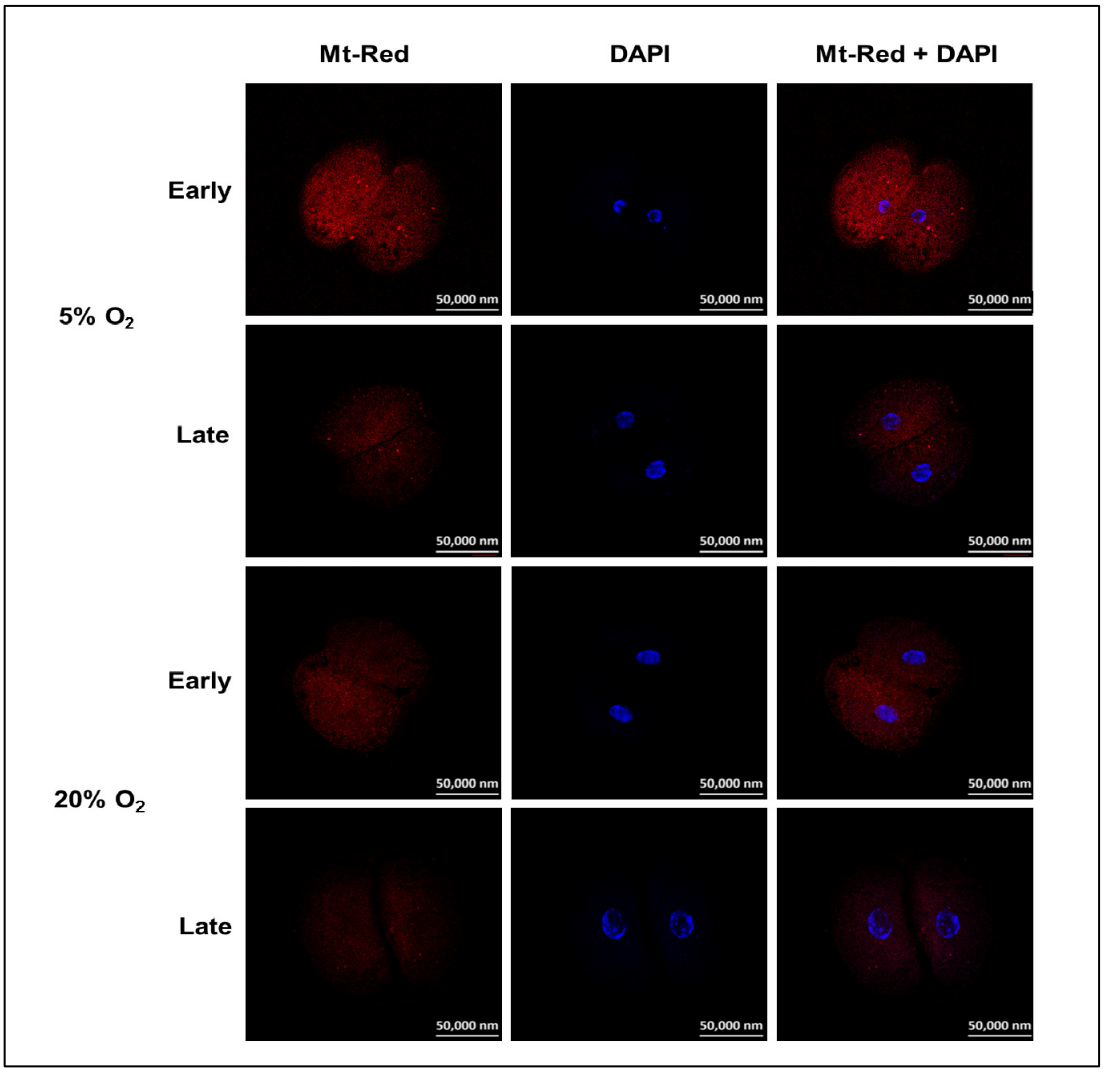


Figure 30: Mitochondrial activity of early vs. late cleaving 2-cell derived from 5% or 20% oxygen culture conditions after staining by MitoTracker (red fluorescent). Nuclei are stained with DAPI (blue). Original magnification 40x. Scale bars, 50 µm.

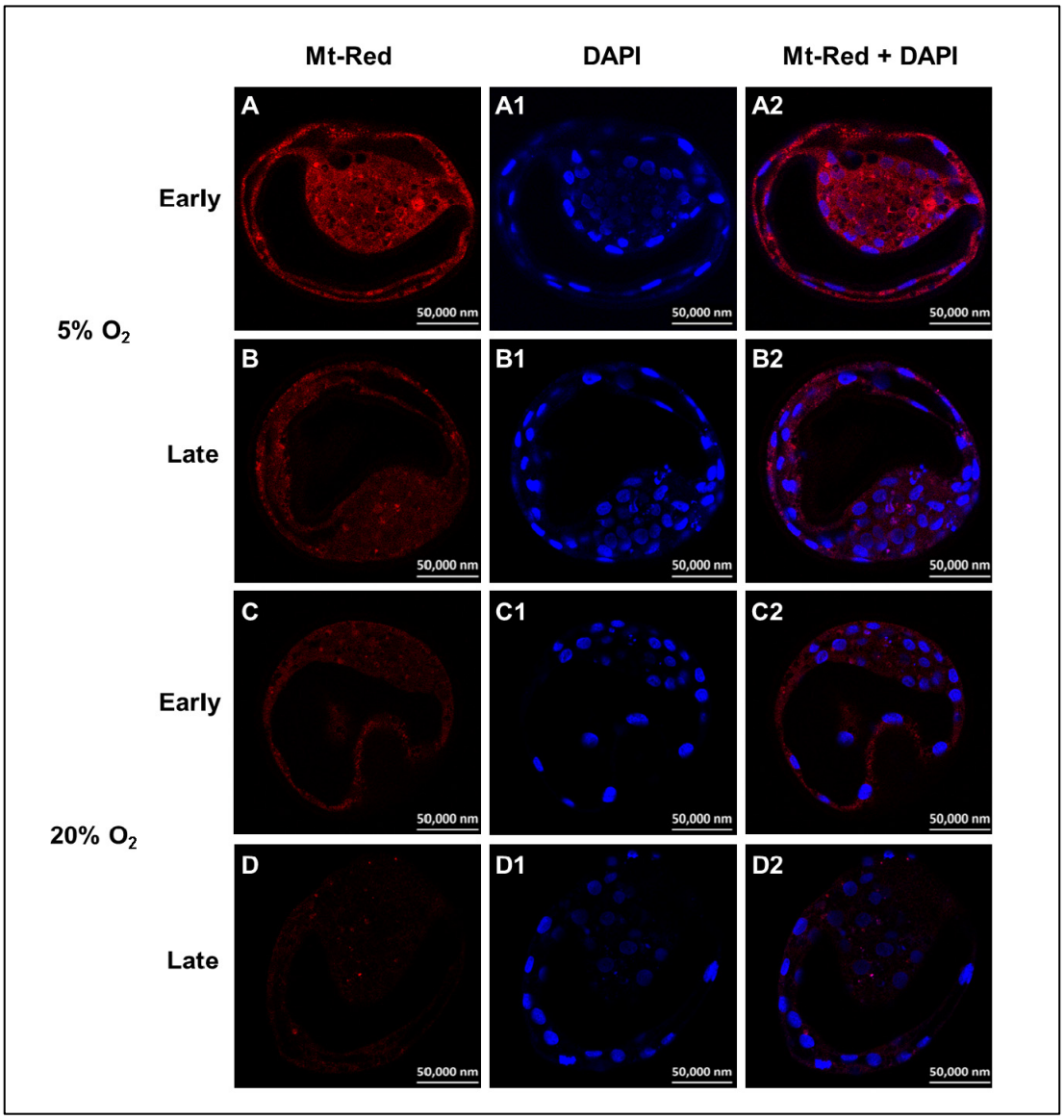


Figure 31: Mitochondrial activity of early and late developing blastocysts derived from 5% or 20% oxygen conditions after staining by MitoTracker (red fluorescent). Nuclei are stained with DAPI (blue). Original magnification 40x. Scale bars, 50 µm.

4.3.7 Embryo metabolism in relation to lipid droplet accumulation

In order to elucidate whether mitochondrial activity differences in early vs. late embryos at both 5% and 20% O_2 level is associated with disturbed embryo metabolism which lead to lipid accumulation, embryos were stained with Oil red O system.

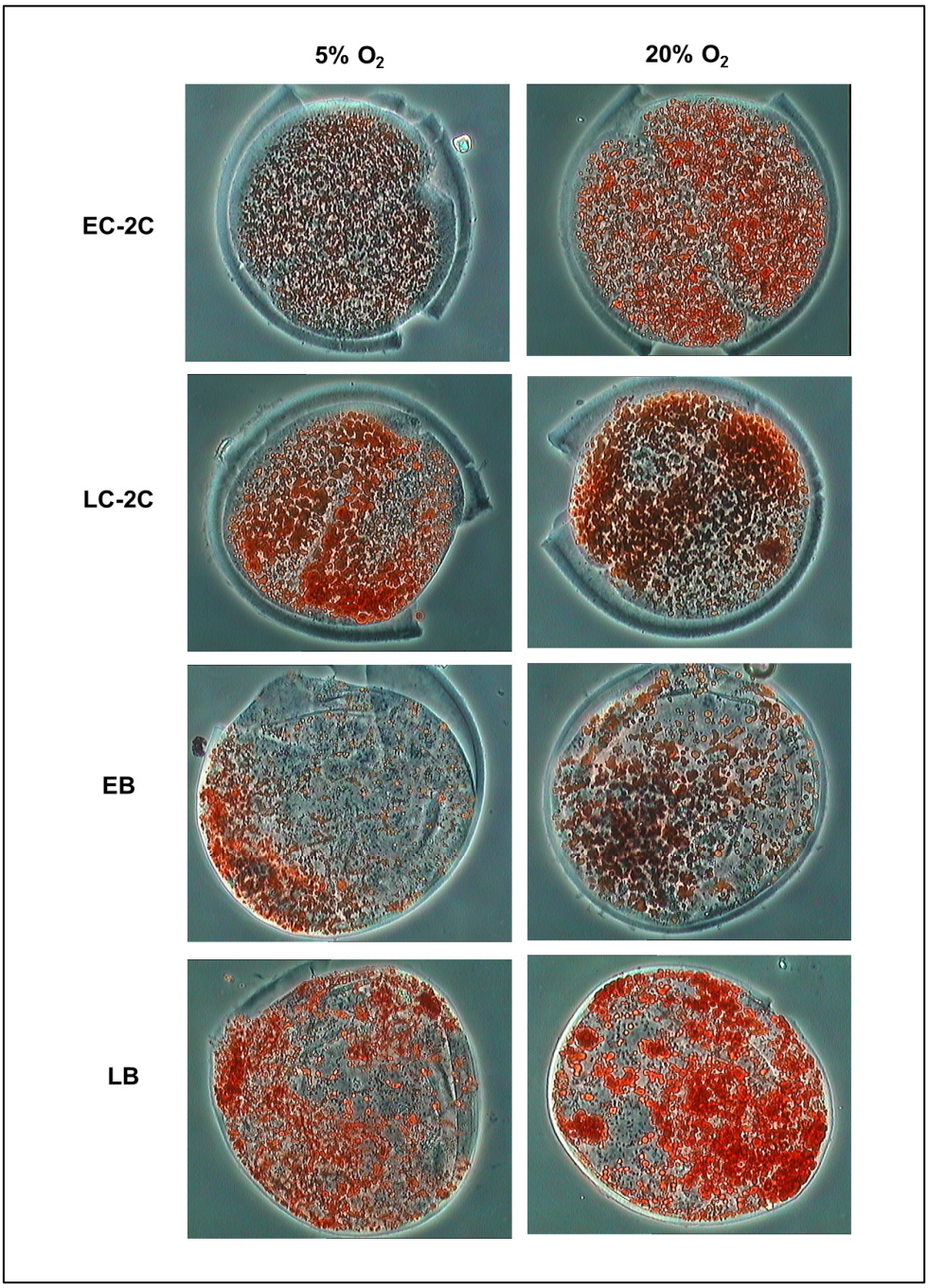


Figure 32: Lipid droplet accumulation as stained by Oil Red O stain in early cleaving 2-cell (EC-2C) vs. late cleaving 2-cell (LC-2C), and early blastocysts (EB) vs. late blastocysts (LB) derived form low (5%) and high (20%) oxygen level culture conditions. Original magnification 40x. Scale bars, 70 µm.

The obtained pictures by light microscopy demonstrate that, lipid accumulation in early blastocysts cultured under 5% and 20% oxygen tensions were lower than the late ones. While no clear differences were observed at 2cell between early vs. late embryos (Figure 32).

4.3.8 Cryotolerance of competent vs. incompetent embryos

In the present study, embryo hatching rate was used as an indicator of embryo survival ability after cryopreservation. To determine the association between the NRF2 activity and embryo survival ability, we compared the hatching rate of competent vs. incompetent blastocyst embryos derived from 5% and 20% oxygen levels culture conditions. The results revealed that the early developing blastocysts had higher hatching rate accompanied by more NRF2 activity (Figure 22 and 24) than the late group after freezing and thawing (Figure 33).

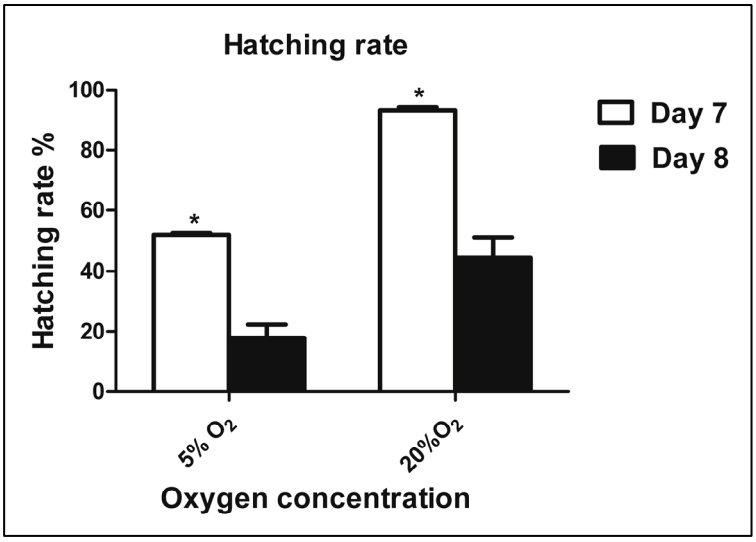


Figure 33: Hatching rate of early and late developing blastocysts derived from 5% or 20% oxygen conditions after vitrification freeze-thaw. Asterisks (*) represent statistically significant differences ($P<0.05$) between early and late developing blastocysts at each oxygen culture condition.

5 Discussion

Compared to the in vivo environment, the in vitro culture conditions are still known for being suboptimal to the bovine preimplantation embryo development, which is manifested by a dramatic change in the transcriptome profile and metabolism of the IVP embryos and subsequently resulted in embryos of poor quality (Gardner et al. 2013, Khurana and Niemann 2000, Lazzari et al. 2002, Lonergan et al. 2003a, Niemann and Wrenzycki 2000, Rizos et al. 2001, Rizos et al. 2002b, Rizos et al. 2002c, Rizos et al. 2003, Thompson et al. 1998). Looking into the differences between in vitro and in vivo environment, in addition to culture media components, there is a significant difference in the oxygen levels, which is relatively higher under in vitro environment. High level of oxygen during in vitro culture is known to be one of the sources of oxidative stress to the embryo. Oxidative stress has been defined as an imbalance between the production of ROS and the activity of antioxidants that are responsible for ROS scavenging from the cells. Hence, cell can overcome the oxidative stress by reducing ROS production through increasing the activity and availability of the antioxidants (Agarwal et al. 2005b, Sies 1986). Thus, understanding the embryo antioxidant adaptive responses may help to design a strategy to decrease the detrimental effect of oxidative stress and improve the embryo developmental competence under in vitro culture conditions. Recently, stage specific exposure of embryos to in vitro or in vivo environment has been shown to result in blastocysts which differ in their transcriptome profile mainly in the activity of NRF2 mediated oxidative stress response pathway (Gad et al. 2012). In order to elucidate embryos reaction to the elevated oxygen tension, keeping the culture environment constant, groups of embryos in different stages of development were exposed to higher or lower oxygen tension in the same vitro culture media condition to investigate the effect of oxidative stress on embryo development and activity of NRF2 mediated oxidative stress response pathway and its association with embryo survival and metabolism.

5.1 High oxygen level in in vitro culture environment induces oxidative stress during early embryo development

Amongst the several factors controlling the embryo production of mammalian embryos, oxygen concentration in in vitro culture environment is considered as a critical

component in all of the in vitro culture systems (Dumoulin et al. 1999, Kitagawa et al. 2004, Luvoni et al. 1996, Thompson et al. 1990). Several studies have compared between culturing embryos in vitro under atmospheric oxygen concentration and low (5%) oxygen level as in oviduct. They showed that culturing embryos in vitro under high oxygen level (20%) creates unfavorable conditions that enhance the generation of ROS and induce the oxidative stress to the embryos (Favetta et al. 2007, Goto et al. 1993). Oxidative stress induced by ROS was previously reported as a promoter for many detrimental effects during the IVP procedure resulting in reduction of embryo quality and delay in embryonic development (Agarwal et al. 2003, Agarwal et al. 2005a, Das et al. 2006, Harvey et al. 2002, Khurana and Niemann 2000, Liu and Keefe 2000, Paszkowski and Clarke 1996). In agreement with that, in the present study, results showed that culturing embryos under high oxygen level (20%) in vitro until blastocyst stage induced accumulation of ROS (Figure 13) which is associated with reduced embryo cell number and blastocyst formation rate (Table 5). On the other hand, reduced environmental oxygen concentration in the culture atmosphere may improve embryo development via improving the energy metabolism (Khurana and Wales 1989). Therefore, three possible ways to minimize oxidative stress damage to embryos were suggested during in vitro production of mammalian embryos process: 1) reducing the oxygen concentration in the gas phase of incubators used for maturation, fertilization and embryo culture; 2) deploying the formulation of embryo culture media to include some elements designed to increase the ability of embryos to fight against oxidative damage mediated by ROS; and 3) decreasing the duration of insemination as much as possible to reduce the harmful effect of oxidative stress damage caused by spermatozoal metabolism (Catt and Henman 2000).

5.2 Activity of NRF2 and its inhibitor KEAP1 in response to oxidative stress

NRF2/KEAP1 system is one of the cell self defense mechanisms against oxidative stress. NRF2 is a transcription factor that works as a system with KEAP1 protein in response to oxidative stress to reduce the ROS level by activating battery of cytoprotective and antioxidant genes through a common DNA regulatory elements, termed (AREs) or the antioxidant response elements (Itoh et al. 1997, Kobayashi et al. 2006, Nguyen et al. 2000, Nguyen et al. 2003). The potential functions of the

NRF2/KEAP1 signaling pathway as cytoprotective system against oxidative stress has been verified using either NRF2 knock-out mice or its cells (Chanas et al. 2002, Cheung et al. 2012, Enomoto et al. 2001, Gong and Cederbaum 2006a, Khor et al. 2008, Ramos-Gomez et al. 2001). Recently, in series of experiments, which have just been published in 2012, designed to investigate the molecular mechanisms and pathways which are involved in bovine embryonic genome activation, it has been identified that NRF2 pathway to be one of the dominant pathways affected when embryos are exposed to various environmental stressors (Gad et al. 2012, Held et al. 2012). However, the role of NRF2 pathway in embryonic development is not well understood. Therefore, in the present study, an in vitro model with high or low oxygen tension was used to investigate the effect of oxidative stress on the activity of NRF2 mediated oxidative stress response pathway during early bovine embryos development.

In response to oxidative stress, in the present study, NRF2 transcript abundance was higher starting from 8-cell stage. However, before 8-cell stage, NRF2 activity was not triggered by oxidative stress, since the embryo is dependent on maternal store of NRF2 pathway related genes transcripts. Therefore, it is evident here that the elevated expression of NRF2 gene at mRNA level in embryos cultured under 20% O_2 as reaction to accumulated ROS is dependent on the activation of embryonic genome. Supporting that, several investigators observed a culture environment-dependent expression of genes involved in bovine embryo differentiation, apoptosis and oxidative stress response after embryonic genome activation, at blastocyst stage (Rizos et al. 2002b, Rizos et al. 2003, Wrenzycki et al. 2001). In addition, higher NRF2 mRNA and protein expressions of embryos at 20% O_2 in this study may reflect an internal response to resist the increased oxidative stress under in vitro conditions. Similar NRF2 response was observed in mammalian cells exposed to different oxidative stress induction factors such as ethanol (Dong et al. 2008), heavy metals (He et al. 2007), cigarette smoke (Knorr-Wittmann et al. 2005) and arachidonic acid (Gong and Cederbaum 2006b).

Under oxidative stress free condition, inactive form of NRF2 is abundant being sequestered by KEAP1 protein in the cytoplasm. Therefore, the ability of NRF2 to activate its downstream antioxidants is regulated by its inhibitor, KEAP1, activity (Dhakshinamoorthy and Jaiswal 2001, Itoh et al. 1999). In response to oxidative stress, cytoplasmic KEAP1, which is acting as a redox sensor, releases NRF2 protein as free molecule to be translocated into the nucleus to activate AREs of many cytoprotective

genes and NRF2 itself (Itoh et al. 2003, Lau et al. 2008, Nguyen et al. 2003, Osburn and Kensler 2008). A recent study provided evidence that knockdown of KEAP1 using siRNA enhanced the NRF2 transcriptional activity in the 36M2 human epithelial ovarian cancer cell line in vitro (Konstantinopoulos et al. 2011). In agreement with this observation, in the present study, a reciprocal NRF2 and KEAP1 gene and protein expression patterns were observed in blastocyst stage embryos exposed to low or high oxygen level.

5.3 Expression of NRF2 antioxidant downstream genes in response to oxidative stress

NRF2 controls several antioxidant pathways (reviewed by Giudice et al. 2010 and Gorrini et al. 2013) including: (1) NADPH production, which is controlled by G6PD (glucose-6-phosphate dehydrogenase), PHGDH (phosphoglycerate dehydrogenase), ME1 (malic enzyme 1) and IDH1 (isocitrate dehydrogenase 1); (2) TXN generation and ultilization, which are regulated by TXN1, TXNRD1 (thioredoxin reductase 1) and PRDX1; (3) GSH production, regeneration and utilization which are controlled by GCLM (glutamate–cysteine ligase complex modifier subunit), GCLC (the GCL catalytic subunit), GSR (glutathione reductase), cystine/glutamate transporter XCT, GPX2 (glutathione peroxidase 2) and GSTs (glutathione S-transferase family incudes GSTA1, GSTA2, GSTA3, GSTA5, GSTM1, GSTM2, GSTM3 and GSTP1). Moreover, additional antioxidant enzymes, which are working together as a system for scavenging ROS from the cell, are also controlled by NRF2 such as SODs and CAT. These groups of antioxidants have complementary and overlapping antioxidant functions and are all upregulated by activation of NRF2 (Giudice et al. 2010, Gorrini et al. 2013, Yeh and Yen 2006, Zhu et al. 2005). In the present study, selected antioxidant genes (SOD1, CAT, PRDX1, HMOX1, NQO1 and TXN1) from different antioxidant pathways regulated by NRF2 were analyzed during specific stage of development in embryos derived from low and high oxidative stress culture conditions. Those embryos exhibited different gene expression patterns under oxidative stress conditions either before or after embryonic genome activation.

5.3.1 Transcript abundance of NRF2 antioxidant downstream genes before embryonic genome activation under oxidative stress conditions

It is a widely accepted fact that the embryo protection against oxidative stress during earlier stages of development, in part, is driven by maternal pool of antioxidant enzymes which were stored in the oocyte as a set of mRNA transcripts and proteins during oogenesis (El Mouatassim et al. 1999, Harvey et al. 1995). In agreement with this, in the present study, abundance of NRF2 antioxidant downstream genes before 8-cell stage was not significantly triggered by oxidative stress. Some of the antioxidants namely SOD1, CAT and PRDX1 showed significantly lower abundance in 2- and 4-cell stage embryos under high oxygen tension compared to those cultured under low (5%) oxygen level. This is maybe due to that the embryo under high oxygen tension utilized more antioxidants of maternal origin to scavenge the accumulated ROS which is important for their development and survival under oxidative stress culture conditions (Agarwal et al. 2005b, El Mouatassim et al. 1999, Harvey et al. 1995).

5.3.2 Transcript abundance of NRF2 antioxidant downstream genes after embryonic genome activation under oxidative stress conditions

Major embryonic genome activation occurs at 8- to 16-cell stage of bovine embryonic development. Before 8-cell stage, the control of early embryonic development gradually shifts from maternal RNAs transcripts and proteins which are stored in the oocyte to gene products which are generated after embryonic genome activation (Memili et al. 1998). Previous results of different studies identified several hundred transcripts with increased abundance in bovine embryos by the time of embryonic genome activation (Held et al. 2012, Misirlioglu et al. 2006, Vigneault et al. 2009). Ontology classification of the differentially expressed genes in these studies showed that they are involved in many cellular functions and pathways including RNA processing, chromatin structure, transcription and protein biosynthesis, signal transduction, cellular response to stress and antioxidant activity. Therefore, the embryo performance during early development is greatly influenced by the activation of its genome. For instance, it has been observed that 8- to 16-cell bovine embryos are more resistant to oxidative stress induced by H_2O_2 than zygotes. These different sensitivities to oxidative stress, before and after the time

of embryonic genome activation, are due to variations in the embryo defense mechanisms during the embryonic development (Morales et al. 1999). In the current study, in line with the expression of NRF2 in those embryos which survived and reached the required stage of development in acceptable developmental time window, the expression of NRF2 downstream antioxidants (SOD1, CAT, PRDX1, HMOX1, NQO1 and TXN1) showed higher expression starting from 8-cell stage onwards in those embryos cultured at high oxygen level. This may reflect the bovine embryo ability to activate NRF2 antioxidant pathway related genes at the time of embryonic genome activation. Moreover, the higher abundance of antioxidants was associated with the presence of active form of NRF2 protein, which is localized in the nucleus to activate antioxidant genes by binding to AREs site (Lau et al. 2008, Nguyen et al. 2003, Osburn and Kensler 2008). Similar observations were also reported by several investigators which showed high antioxidant activity for many antioxidant genes such as CAT and SOD1 (Dreger et al. 2009, Yeh and Yen 2006, Zhu et al. 2005), HMOX1 and NQO1 (Ade et al. 2009, Ben-Dor et al. 2005, Nerland 2007), PRDX1 (Kim et al. 2007), TXN1 (Im et al. 2012) and SOD2 and GPX3 (Konstantinopoulos et al. 2011) as a result of increased NRF2 activity in the cell. In addition, pretreatment with the NRF2 inducer, 3H-1,2 dithiole-3-thione (D3T), significantly induced NRF2 protein expression and NRF2-ARE binding in mouse embryos. As a result, the mRNA expression of NRF2 downstream target genes including SOD1, SOD2, SOD3, CAT, GSR, TXN1, TXN2, GSH and GPX1 and 3 was strongly increased. It also improved the levels and the activities of the antioxidant enzymes and significantly decreased the ROS levels and apoptosis in mouse embryos. These results suggested that NRF2-mediated oxidative stress response is involved in the induction of antioxidant response and may serve as a shield to protect the embryo against oxidative stress during embryo development in mouse (Dong et al. 2008, Harris and Hansen 2012).

5.4 Oxidative stress induces lipid accumulation during early bovine embryo development

In addition to the genes associated with NRF2 mediated oxidative stress response pathway, several lipid metabolism genes were also found to be affected by stage-specific alternate in vitro or in vivo culture of bovine embryos (Gad et al. 2012). Recent

reports also suggested a potential involvement for NRF2 in lipid metabolism as well as lipid accumulation in mouse liver (Huang et al. 2010, Okada et al. 2009, Tanaka et al. 2008). Accumulation of lipid is one of the major problems in IVP embryos which lead to a reduced cryotolerance after freezing and thawing (Abe et al. 2002). Exposure of embryos to high oxygen tension resulted in up-regulation of SREBP1 gene and its downstream lipid biosynthesis gene ACACA1 beyond 8-cell stages (Figure 18). SREBF1 is known to be activator for many genes involved in lipid biosynthesis (FAS and ACACA1). Several studies previously observed more SREBF1 activity under oxidative stress conditions that lead to induce lipid accumulation in different mammalian cells (Eberle et al. 2004, Ferre and Foufelle 2007, Matsuzawa et al. 2007, Sekiya et al. 2008, Tanaka et al. 2008). On the other hand, CPT2 and PPARAα which are involved in lipid oxidation pathways were also up regulated in the present study as a response to the increased oxidative stress, but it may not have been sufficient to break down the excessive lipid accumulation under embryo in vitro culture condition.

The reasons for accumulation of lipid in in vitro produced embryos can be either due to application of serum as an external source of excess lipid (Ferguson and Leese 1999, McEvoy et al. 2000, McEvoy et al. 2001) or the inability of the embryo to metabolize the available lipid due to disturbed mitochondrial activity as a result of excessive accumulation of ROS (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007a). The later could be evidenced in the present study in which embryos from the same in vitro culture condition showed significant differences in the accumulation of ROS (Figure 13), mitochondrial activity (Figure 19) and lipid accumulation (Figure 20) under high oxygen tension culture condition.

5.5 Embryo survival under oxidative stress condition is associated with the activity of NRF2 mediated oxidative stress response pathway

The findings of the first part of this work evidenced stage dependent variation in NRF2/KEAP1 response mechanism of embryos to environmental oxidative stress. Since this analysis was performed using embryos which have survived and reached to the required stage of development in acceptable time window, it was not possible to know the status of NRF2 defense mechanism of those embryos which did not survive and reached to the required stage of development under oxidative stress condition. Thus, in

order to associate embryo's NRF2 mediated oxidative stress response to its developmental potential and survival under oxidative stress condition, developmental rates of first cleavage and blastocyst formation were used as a metric to distinguish between embryos with high or low competence for further development. This model has been used in several species due to the fact that early developing 2-cell and early blastocyst embryos are competent to induce higher rate of successful pregnancy after transfer as compared to their late developing counterparts (Barrenetxea et al. 2005, Bernardi and Delouis 1996, Grisart et al. 1994, Hasler et al. 1995, Hasler 2000, Lee et al. 2012, Lonergan et al. 1999, Monson et al. 1992, Muthukumar et al. 2013, Plante et al. 1994, Shapiro et al. 2001). These differences in developmental competence are found to be associated with the alteration in expression of developmentally important genes associated with various embryonic physiological processes (Brevini et al. 2002, Dode et al. 2006, Fair et al. 2004). Therefore, in addition to the activity of NRF2 mediated oxidative stress response and its cytoplasmic inhibitor KEAP1, selected NRF2 antioxidant target genes (CAT, PRDX1, SOD1 and TXN1) were evaluated in early cleaving 2-cell and early blastocyst embryos (as developmentally competent embryos) vs. late cleaving 2-cell and late blastocysts (as incompetent embryos). At 2-cell stage, the transcripts of NRF2, its antioxidant targets and the cytoplasmic inhibitor *KEAP1* seem to be inherited from the maternal mRNA which is accumulated during oogenesis. In addition, the decreased mRNA level of those genes which was observed in the early 2-cell embryos under high oxygen level may reflect better efficiency to use the stored maternal transcripts before the embryonic genome activation. This may also explain the reduced ROS level in the early 2-cell embryos under high oxidative stress conditions (Figure 29) which may contribute to enhance the cleavage processes of those embryos earlier than their late counterparts. At blastocyst stage, early developing or competent embryos showed higher activity of NRF2 which expressed as mRNA and nuclear active protein. The abundance of NRF2 protein in the nucleus is accompanied by higher abundance of antioxidant genes (CAT, PRDX1, SOD1 and TXN1) in early competent blastocysts (Figure 27) and subsequently resulted in reduced ROS accumulation (Figure 29). A similar study showed the association of antioxidants expression and ROS accumulation in early cleaving competent and late cleaving non competent 2-cell stage embryos (Held et al. 2012). In addition, several studies have evidenced that translocation of NRF2 protein into the nucleus has been shown to

increase the expression of antioxidants, which have been tested in the current study, including PRDX1 (Kim et al. 2007, Ohta et al. 2008), TXN1 (Li et al. 2010), CAT and SOD1 (Dong et al. 2008). In contrary, loss of NRF2 functions leads to reduction of the activity of many antioxidants and induce oxidative stress in cells, which is indicated by increased intracellular ROS levels and resulted in early embryonic cell death (Leung et al. 2003). Taken together, the results strongly suggested that embryo ability to survive and develop under oxidative stress condition is associated with its ability to activate NRF2 mediated defense mechanism which is playing a vital role for activation of antioxidant defenses to scavenge the accumulated ROS.

5.6 Mitochondrial activity is associated with the activity of NRF2

Mitochondria are known to play a vital role in early embryo metabolism and their subsequent function is associated with developmental competence (Barnett and Bavister 1996, Leese 2012, Tarazona et al. 2006, Wilding et al. 2001). Oxidative stress induced by ROS accumulation has been implicated as a causative factor for mitochondrial deficiency (Cui et al. 2012, Kowaltowski and Vercesi 1999, Milei et al. 2001, Shokolenko et al. 2009). In the present study, early competent 2-cell and blastocyst embryos, which have lower ROS accumulation, showed higher mitochondrial activity than their late non-competent counterparts with elevated ROS accumulation. These results were in agreement with the findings of Tarazona et. al., (2006) in bovine which showed high mitochondrial activity levels in the competent embryos. So far, this study has established a direct relationship between embryo developmental competence and activation of NRF2 mediated oxidative stress response pathway, reduced ROS accumulation and active mitochondria at blastocyst stage. This notion is supported by several studies which confirmed the fact that NRF2 mediated oxidative stress response is postulated to play a role in the protection of mitochondria against oxidative stress damages and regulation of the mitochondrial biogenesis (Greco and Fiskum 2010, MacGarvey et al. 2012, Piantadosi et al. 2008, Ungvari et al. 2011). In addition, activities of several NRF2 antioxidant downstream genes, such as SOD1, PRDX1 and CAT, were previously observed in mitochondria and suggested to reduce the ROS accumulation and oxidative injury to the mitochondria (Bai et al. 1999, Bai and Cederbaum 2001, Okado-Matsumoto and Fridovich 2001). While in the early 2-cell

embryos, high mitochondrial activity maybe due to the activity of the maternal inherited antioxidants or depends on another antioxidant systems. The activity of the different antioxidant mechanisms may only turn on during specific stages of development (Hansen 2012).

Mitochondria are the power manufacture of the cells. They are generating energy as an ATP from different sources via a set of metabolic processes such as fatty acid beta oxidation. Recently, it has been shown that addition of L-carnitine and ammonium compound to the culture media improved the fatty acid translocation into the mitochondria and initiated the lipid beta oxidation process, as a result, the mitochondrial activity was increased and the cleavage rate was improved in porcine embryos (Somfai et al. 2011). The CPT2 transcript, which is responsible for the fatty acid translocation into the mitochondrial matrix for beta oxidation process, is found to be highly abundant in competent blastocysts, which showed reduced lipid droplets after Oil red O staining. In addition, recent NRF2 knockout mouse model studies suggested a potential role for NRF2 in hepatic lipid accumulation in liver (Huang et al. 2010, Tanaka et al. 2008). However, direct association of NRF2 mediated oxidative stress response pathway with the bovine embryo metabolism in general and lipid metabolism in particular needs further investigation.

5.7 Cryotolerance of embryos is associated with their NRF2 activity

The embryo capability to an outstanding cryopreservation with limited loss in viability is indispensable to success of the final outcome of in vitro production technique. At present, embryos that have been cryopreserved show greatly reduced viability following thaw and transfer compared with the fresh embryos that have not been cryopreserved. It is plausible that cryopreservation may raise the rates of lipid peroxidation in cell membranes as a result of an increase in ROS levels. Therefore, cryopreservation process is considered to be one of the main inducers for oxidative injury during in vitro production of mammalian embryos, which decrease the developmental competence after embryo thawing (Lane et al. 2002, Rahimi et al. 2003, Zhao et al. 2012). In gametes, it has been demonstrated that levels of antioxidants are reduced following cryopreservation (Bilodeau et al. 2000, Marti et al. 2008). In addition to the beneficial effect of culture media supplementation with antioxidants to the early embryo

development, there has been a significant improvement in the quality in form of ability of those embryos to survive after cryopreservation (Goto et al. 1992, Nasresfahani et al. 1990a, Noda et al. 1991, Nonogaki et al. 1991, Nonogaki et al. 1992, Tarin and Trounson 1993). In the present study, hatching rate of the early competent vs. late incompetent blastocysts was evaluated as an indicator for the embryo viability and ability to survive after cryopreservation. Developmentally competent blastocysts derived from both oxygen levels showed a better hatching rate after cryopreservation than their non-competent counterparts. This may be attributed to activated NRF2 oxidative stress response in competent embryos which enable them to overcome the oxidative stress induced by the cryopreservation procedure (Lane et al. 2002, Rahimi et al. 2003, Zhao et al. 2012).

In conclusion, this study has demonstrated the potential role of NRF2-mediated oxidative stress response pathway activity in bovine preimplantation embryo development and survival under oxidative stress condition for the first time. Moreover, results of the present study support the hypothesis that the fate of early bovine embryo under suboptimal environmental condition is associated with its ability to activate the oxidative stress defense mechanism, which is mainly driven by NRF2 mediated stress response pathway. The findings of the present study are summarized schematically to show the relationship between the activity of the NRF2-mediated oxidative stress defense mechanism and embryo developmental competence under suboptimal culture condition (Figure 34).

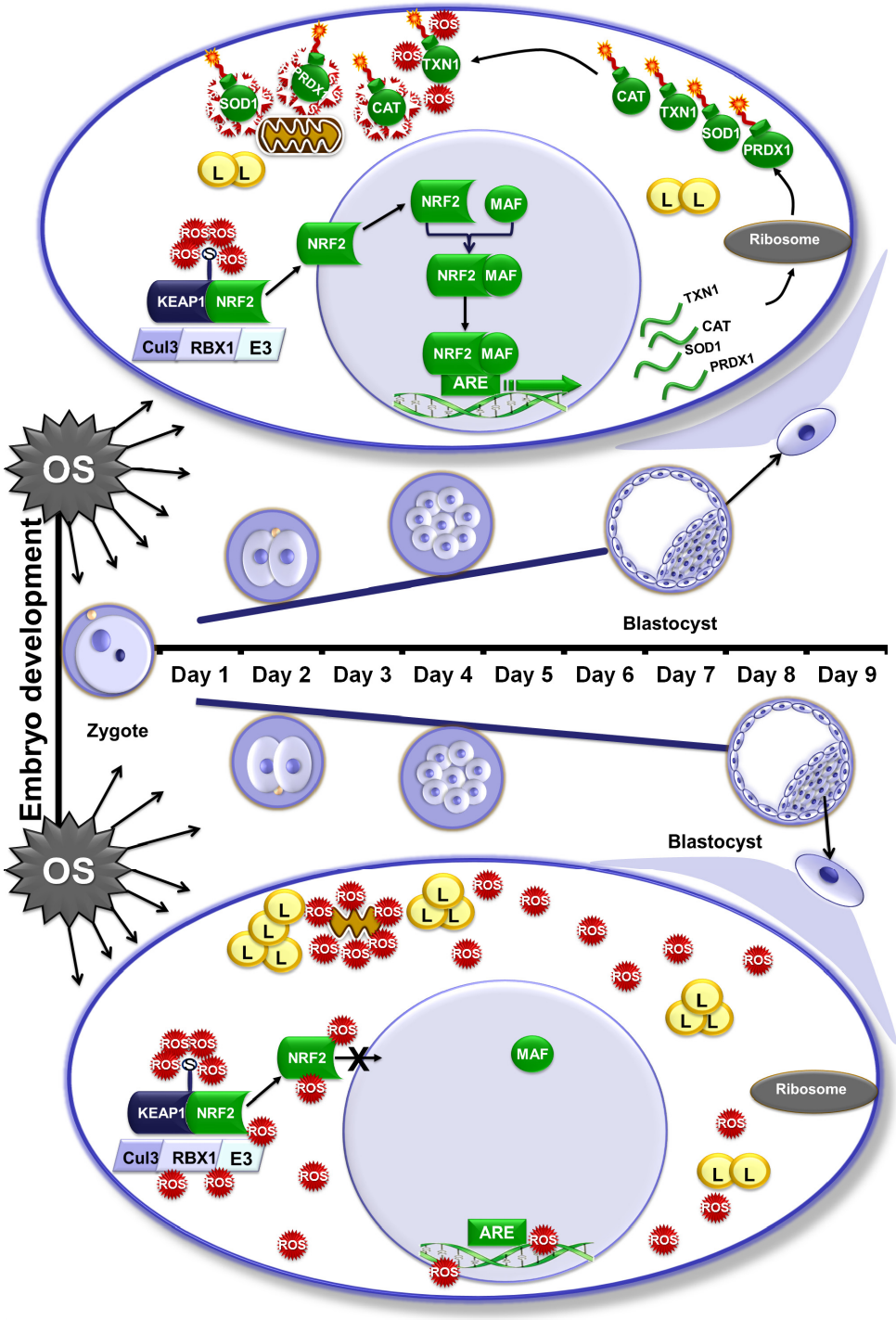


Figure 34: Hypothetical model of the NRF2-mediated oxidative stress response pathway in competent (embryos that reach the blastocyst stage at 7 days pi) and incompetent (embryos that reached the same stage at 8 days pi) under a suboptimal culture environment. Competent blastocysts have an active NRF2 protein that is localized in the nucleus, where it binds to the antioxidants reactive elements (AREs) in the promoters of

antioxidant genes. This activates antioxidant gene expression so the respective proteins can scavenge the ROS and subsequently maintain the activity of mitochondria for better metabolism of lipids (L). In the case of incompetent embryos, however, inactive NRF2 leads to lower levels of antioxidants and subsequent accumulation of ROS, which impair the activity of mitochondria and embryo metabolism. As a result of this disturbed metabolism, lipid (L) accumulates in those embryos.

6 Summary

IVP of bovine embryos is one of the most important tools to improve the genetic potential of cattle. Moreover, it has been used to study the biology and molecular mechanism of embryogenesis. During IVP of bovine embryos, high oxidative stress can negatively affect oocyte maturation, fertilization and early embryo development. Thus, the protection against oxidative stress is one of the mechanisms of the embryo to survive under suboptimal environment. NRF2 is a key transcriptional factor that binds with AREs to activate battery of antioxidant genes and tackle the oxidative stress. Therefore, in the present study, two experiments have been preformed to investigate the association between NRF2 mediated oxidative stress response activity during the early embryonic development and the ability of the in vitro produced bovine embryos to survive under oxidative stress conditions. The aim of the first experiment was to examine whether the pre-implantation bovine embryos are able to activate NRF2-mediated oxidative stress response, its downstream antioxidant genes (CAT1, HMOX1, NQO1, PRDX1, SOD1 and TXN1) and genes that control lipid metabolism and believed to be regulated by NRF2 (SREB1, ACACA, CPT2 and PPARAα) under oxidative stress conditions. In addition, in blastocyst stage embryos, the effect of oxidative stress on the generation of ROS and their implications on embryonic metabolism, in terms of mitochondrial activity and lipid content, have been observed. For this, oocytes collected from slaughter house ovaries were in vitro matured, in vitro fertilized and in vitro cultured under 5% or 20% oxygen level culture conditions. Following this, the 2-, 4-, 8-, 16-cell and blastocyst stages embryos were collected at 32, 48, 72, 92 and 168-192 hpi from each treatment group. Three biological replicates, each containing fifty 2-cell, thirty 4-cell, twenty 8-cell, fifteen 16-cell and ten blastocyst stage embryos were used for RNA isolation using PicoPure RNA isolation kit (Arcturs, Munich, Germany). Following this, cDNA synthesis was performed in 20 µl of total reaction volume. Gene expression level of NRF2 and its downstream target genes, NRF2 inhibitor (KEAP1) and genes involved in lipid metabolism were measured using qPCR. The data analysis was then performed using relative standard curve method using GAPDH as internal normalizer. In addition, immunohistochemistry was used to detect spatial protein expression of NRF2 and KEAP1 in the blastocyst stage embryos. Results of the first experiment showed that, as a response to induced oxidative stress conditions, blastocysts cultured under 20% oxygen exhibited higher ROS level than the

5% oxygen groups. In addition, the mRNA expression level of NRF2 was up-regulated while the expression level of its inhibitor, KEAP1, was reduced in 8-cell, 16-cell and blastocyst stages embryo cultured under 20% compared to 5% oxygen level. A similar trend of NRF2 and KEAP1 protein expressions was confirmed at the blastocyst stage. Moreover, the relative abundance of the NRF2 downstream target antioxidant genes (CAT1, HMOX1, NQO1, PRDX1, SOD1 and TXN1) and genes involved in lipogenic pathways (SREB1, ACACA, CPT2 and PPARAα) were found to be higher ($P < 0.05$) in 8-cell to blastocyst stage embryos from 20% oxygen culture groups. On the other hand, the relative abundance of some antioxidants (CAT, PRDX1 and SOD1) was significantly higher from 2-cell until 8-cell stage in 5% compared to the 20% oxygen level groups. In general, results of this experiment indicated that culturing bovine embryos under high oxygen tension resulted in activation of NRF2 mediated oxidative stress response and lipogenic pathways. However, the possible interaction between NRF2 mediated oxidative stress response and lipogenic pathways and its association with embryo lipid metabolism need further investigation. Also, embryos cultured under high (20%) oxygen level exhibited low mitochondrial activity, high lipid accumulation and low blastocyst rates compared to low (5%) oxygen group which reflected the detrimental effect of elevated ROS levels on the embryo metabolism and developmental competence under high oxidative stress conditions.

In order to know whether the ability of early bovine embryos to survive under oxidative stress condition is in relation to their NRF2-mediated oxidative stress response pathway activity, in the second experiment, the expression of NRF2, KEAP1 and antioxidant genes in early versus late cleaving 2-cell and blastocyst stage embryos cultured under high or low oxidative stress conditions was evaluated. For this, embryos obtained from the in vitro culture before 32 hpi were considered as developmentally competent 2-cell stage embryos, while the 2-cell embryos obtained after 36 hpi were considered as developmentally incompetent embryos. Likewise, the development of the blastocyst stage embryos was evaluated at day 7 and 8 pi. The early developing blastocysts (D7 pi) were considered as competent embryos, while the embryos which reached to the same stage at day 8 pi were categorized as incompetent embryos.

Embryos from each competence category (competent vs. incompetent) which were generated from high (20%) or low (5%) oxygen level were used to investigate the expression level of NRF2, KEAP1, NRF2 downstream antioxidant genes (CAT,

PRDX1, SOD1 and TXN1) and genes related to lipid metabolism (SREBP1 and CPT2). Moreover, the NRF2 transcriptional activity in competent vs. incompetent embryos was assessed by evaluating the amount and distribution of NRF2 and KEAP1 proteins using immunofluorescence technique. Furthermore, the level of ROS, mitochondrial activity and lipid content were determined for embryos from each tested category. Finally, the ability of the competent and incompetent blastocysts to survive after cryopreservation (in terms of hatching rates) was evaluated. Results showed that NRF2 and its antioxidant target genes were found to be increased in early compared to late blastocyst stage embryos. Similarly, more active nuclear NRF2 protein has been observed in early or competent embryos compared to the late ones either under high or low oxygen level culture conditions. This was accompanied by reduced levels of ROS in the early developing embryo compared to the late ones. Moreover, competent embryos exhibited higher mitochondrial activity and lower lipid accumulation than the incompetent ones under both 5% and 20% O_2 culture conditions. Subsequently, the viability of the competent embryos after cryopreservation, in terms of hatching rates, was higher. In conclusion, this study demonstrated that under oxidative stress conditions, pre-implantation bovine embryos are able to activate the NRF2-mediated oxidative stress response pathway, which is found to be associated with their survival under in vitro culture conditions.

7 Zusammenfassung

In der in vitro Produktion von bovinen Embryonen kann ein zu hoher oxidativer Stress einen negativen Effekt auf die Eizellmaturation, die Fertilisation und die frühe embryonale Entwicklung haben. Deswegen ist der Schutz der Embryonen vor oxidativen Stress einer der embryonalen Mechanismen unter suboptimalen Entwicklungsbedingungen zu überleben. NRF2 ist der entscheidende Transkriptions Faktor welcher mit dem AREs bindet um die Kaskade der Antioxidans-Zielgene zu aktivieren und den oxidativen Stress zu bewältigen. Deshalb war das Ziel der Studie, die Assoziation zwischen der NRF2-vermittelten oxidativen Stressreaktion während der frühen Embryonalentwicklung und die Überlebensfähigkeit der in vitro produzierten bovinen Embryonen unter oxidativen Stresskonditionen zu untersuchen. Das Ziel des ersten Experiments war es festzustellen, ob pre-implantative bovine Embryonen unter oxidativen Stressbedingungen in der Lage sind die NRF2-vermittelte oxidative Stressantwort und deren nachgeschalteten Antioxidans-Gene (CAT1, HMOX1, NQO1, PRDX1, SOD1 und TXN1) sowie Gene die den Lipidmetabolismus kontrollieren und ebenfalls durch NRF2 reguliert werden (SREB1, ACACA, CPT2 und PPARAα), zu aktivieren. Zusätzlich sollte im Blastozytenstadium der Effekt des oxidativen Stress auf die Generierung von ROS und dessen Auswirkungen auf den embryonalen Metabolismus in Bezug zur Mitochondrien-Aktivität und der Lipidzusammensetzung beobachtet werden. Dafür wurden Eizellen von Ovarien von Schlachthoftieren in vitro maturiert, in vitro fertilisiert und in vitro unter den Kulturbedingungen von 5% und 20% Sauerstoff kultiviert. Nachfolgend wurden 2-, 4-, 8-, 16-Zell- und Blastozystenstadien zu den Zeitpunkten 32, 48, 72, 92 und 168-192 hpi der jeweiligen Versuchsgruppen gesammelt. Drei biologische Wiederholungen wurden verwendet und jede umfasste fünfzig 2-Zell-, dreißig 4-Zell-, zwölf 8-Zell-, fünfzehn 16-Zell- und zehn Blastozystenembryonen, die dann für die RNA Isolierung mittels PicoPure RNA Isolationskit (Arcturs, Munich, Germany) eingesetzt wurden. Im Anschluss daran wurde die cDNA Synthese mit einem gesamt Reaktionsvolumen von 20 µl durchgeführt. Das Genexpressionsniveau von NRF2, dessen nachgeschalteten Zielgenen, dem NRF2 Inhibitor (KEAP1) sowie Genen die im Lipidmetabolismus involviert sind, wurden mittels qPCR bestimmt. Die Datenanalyse wurde mit der relativen Standardkurvenmethode unter Verwendung von GAPDH zur Normalisierung

durchgeführt. Zusätzlich wurde die Proteinexpression von NRF2 und KEAP1 im Blastozystenstadium mittels Immunhistochemie analysiert. Aus dem ersten Experiment ging als Reaktion auf den induzierten oxidativen Stress eine höhere ROS Aktivität von Blastozyten der 20% Sauerstoffgruppe im Vergleich zu der 5% Sauerstoffgruppe hervor. Zusätzlich war das mRNA Expressionsniveau von NRF2 hoch reguliert, wohingegen die Expression des Inhibitors KEAP1 in 8-, 16-Zell- und Blastozystenstadien aus der 20% Sauerstoffgruppe im Vergleich zu der 5% Gruppe runter reguliert war. Ein ähnlicher Trend von NRF2 und KEAP1 Proteinexpression wurde im Blastozytenstadium bestätigt. Darüber hinaus, zeigten die NRF2 nachgeschalteten Antioxidans-Gene (CAT1, HMOX1, NQO1, PRDX1, SOD1 und TXN1) und Gene aus den lipogenen Signalwegen (SREB1, ACACA, CPT2 und PPARAα) eine höhere signifikante Veränderung ($P < 0.05$) im 8-Zell- zum Blastozystenstadium in der 20% Sauerstoffgruppe. Die relative Veränderung mancher Antioxidants-Gene (CAT, PRDX1 und SOD1) war signifikant höher im 2-Zell- bis 8-Zellstadium in der 5% Sauerstoffgruppe im Vergleich zu der 20% Sauerstoffgruppe. Im Allgemeinen zeigen die Ergebnisse dieses Experiments, dass das Kultivieren von Embryonen unter hoher Sauerstoffkonzentration in einer Aktivierung der NRF2-vermittelter oxidativen Stressreaktion und der lipogenen Signalwege resultiert. Allerdings sind weitere Untersuchungen über die positive Interaktion zwischen NRF2-vermittelter oxidativer Stressreaktion und den lipogenen Signalwegen sowie über die Assoziation mit dem embryonalen Lipidmetabolismus notwendig. Ebenfalls zeigten Embryonen aus der höheren Sauerstoffgruppe (20%) geringe Mitochondrien-Aktivität, hohe Lipidakkumulation und geringe Blastozystenraten im Vergleich zu den Embryonen aus der geringeren (5%) Sauerstoffgruppe. Dieses Ergebnis reflektiert den nachteiligen Effekt des erhöhten ROS Spiegels auf den embryonalen Metabolismus und die Entwicklungskompetenz unter hohen oxidativen Stressbedingungen.

Um festzustellen, ob die Überlebensfähigkeit der frühen bovinen Embryonen unter oxidativen Stressbedingungen in Relation zur ihrer NRF2-vermittelten oxidativen Stressreaktion steht, wurde im zweiten Experiment die Expression von NRF2, KEAP1 und Antioxidans-Genen in früh gegenüber spät entwickelten 2-Zell- und Blastozystenstadien zum einen unter hohen und zum anderen unter niedrigeren oxidativen Stressbedingungen untersucht. Dafür wurden diese Embryonen aus dem in vitro Kulturmedium vor dem Zeitpunkt 32hpi gewonnen und als kompetente 2 Zell-

Stadien Embryonen bezeichnet. Die 2 Zell-Stadien nach 36hpi wurden als inkompetente Embryonen angesehen. Ebenfalls wurde die Entwicklung von Blastozysten nach 7 und 8 pi Tagen beurteilt. Die früh entwickelten Blastozyten (D7 pi) wurden als kompetente Embryonen, die nach 8 pi Tagen dasselbe Stadium erreichten als inkompetente Embryonen beurteilt.

Embryonen aus jeder Kategorie (kompetent vs. inkompetent), die unter hoher (20%) oder niedriger (5%) Sauerstoffkonzentration generiert wurden, wurden für die Expressionsanalyse von NRF2, KEAP1, NRF2 nachgeschalteten Antioxidans-Genen (CAT, PRDX1, SOD1 und TXN1) sowie von Genen des Lipidmetabolismus (SREBP1 und CPT2) eingesetzt. Darüber hinaus wurde die transkriptionelle Aktivität der kompetenten vs. inkompetenten Embryonen durch die Mengenbestimmung und -Verteilung von NRF2 und KEAP1 Proteinen mittels Immunfluoreszenz Technik bewertet. Weiterhin wurden von den Embryonen aus jeder Kategorie die ROS Aktivität, die Mitochondrien-Aktivität und der Lipidgehalt bestimmt. Zum Schluss wurde noch die Überlebensfähigkeit der kompetenten und inkompetenten Blastozyten nach der Kryokonservierung (hinsichtlich der Schlüpfrate) ermittelt. Die Ergebnisse zeigten, dass NRF2 und dessen Antioxidans-Zielgene erhöht in frühen im Vergleich zu späteren Blastozytenstadien vorkamen. Gleichermaßen wurde mehr aktives nukleares NRF2 Protein in frühen oder kompetenten Embryonen im Vergleich zu späteren Embryonen, egal ob diese unter hohen oder niedrigeren Sauerstofflevel kultiviert wurden, ermittelt. Dieses wurde begleitet durch eine reduzierte ROS Aktivität in früh entwickelten Embryonen im Vergleich zu spät entwickelten. Weiterhin zeigten kompetente Embryonen höhere Mitochondrien-Aktivität und geringere Lipidakkumulation als die inkompetenten Embryonen unter beiden Sauerstoffkulturbedingungen. Die Überlebensfähigkeit der kompetenten Embryonen nach der Kryokonservierung war hinsichtlich der Schlüpfrate höher. Diese Studie zeigte, dass unter oxidativen Stresskonditionen pre-implantative bovine Embryonen in der Lage sind den NRF2-vermittelten oxidativen Stressreaktionsweg zu aktivieren, der mit der Entwicklungskompetenz unter in vitro Kulturbedingungen assoziiert ist.

8 References

Abe H, Yamashita S, Satoh T, Hoshi H (2002): Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Molecular Reproduction and Development 61, 57-66

Abecia JA, Forcada F, Zuniga O (2002): The effect of melatonin on the secretion of progesterone in sheep and on the development of ovine embryos in vitro. Veterinary Research Communications 26, 151-158

Abedelahi A, Salehnia M, Allameh AA, Davoodi D (2010): Sodium selenite improves the in vitro follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxide activity. Human Reproduction Update 25, 977-985

Ade N, Leon F, Pallardy M, Peiffer JL, Kerdine-Romer S, Tissier MH, Bonnet PA, Fabre I, Ourlin JC (2009): HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: role of the Keap1/Nrf2 pathway. Toxicological Sciences 107, 451-460

Agarwal A, Allamaneni SS, Nallella KP, George AT, Mascha E (2005a): Correlation of reactive oxygen species levels with the fertilization rate after in vitro fertilization: a qualified meta-analysis. Fertility and Sterility 84, 228-231

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

Agarwal A, Gupta S, Sharma RK (2005b): Role of oxidative stress in female reproduction. Reproductive Biology and Endocrinology 3, 28

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

Agarwal A, Saleh RA, Bedaiwy MA (2003): Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and Sterility 79, 829-843

Agca Y, Monson RL, Northey DL, Mazni OA, Schaefer DM, Rutledge JJ (1998): Transfer of fresh and cryopreserved IVP bovine embryos: Normal calving, birth weight and gestation lengths. Theriogenology 50, 147-162

Aggarwal A, Misro MM, Maheshwari A, Sehgal N, Nandan D (2010): N-Acetylcysteine counteracts oxidative stress and prevents hCG-induced

apoptosis in rat leydig cells through down regulation of caspase-8 and JNK. Molecular Reproduction and Development 77, 900-909

Aiken CEM, Cindrova-Davies T, Johnson MH (2008): Variations in mouse mitochondrial DNA copy number from fertilization to birth are associated with oxidative stress. Reproductive Biomedicine Online 17, 806-813

Aikens J, Dix TA (1990): Mechanisms of lipid-peroxidation initiation. Abstr Pap Am Chem S 200, 281-ORGN

Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K, Richardson DW (1992): Differential contribution of leukocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. Journal of Reproduction and Fertility 94, 451-462

Aitken RJ, Buckingham DW, Brindle J, Gomez E, Baker HWG, Irvine DS (1995): Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. Human Reproduction 10, 2061-2071

Al-Gubory KH, Fowler PA, Garrel C (2010): The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. International Journal of Biochemistry & Cell Biology 42, 1634-1650

Ali AA, Bilodeau JF, Sirard MA (2003): Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology 59, 939-949

Ali J, Whitten WK, Shelton JN (1993): Effect of culture systems on mouse early embryo development. Human Reproduction 8, 1110-1114

Alvarez JG, Minaretzis D, Barrett CB, Mortola JF, Thompson IE (1996): The sperm stress test: A novel test that predicts pregnancy in assisted reproductive technologies. Fertility and Sterility 65, 400-405

Alvarez JG, Storey BT (1992): Evidence for increased lipid peroxidative damage and loss of superoxide-dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. Journal of Andrology 13, 232-241

Alvarez JG, Storey BT (1983): Taurine, hypotaurine, epinephrine and albumin inhibit lipid-peroxidation in rabbit spermatozoa and protect against loss of motility. Biology of Reproduction 29, 548-555

Anand T, Kumar D, Chauhan MS, Manik RS, Palta P (2008): Cysteamine supplementation of in vitro maturation medium, in vitro culture medium or both media promotes in vitro development of buffalo (Bubalus bubalis) embryos. Reproduction Fertility and Development 20, 253-257

Antczak M, Van Blerkom J (1999): Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. Human Reproduction 14, 429-447

Arnheim N, Cortopassi G (1992): Deleterious mitochondrial-DNA mutations accumulate in aging human tissues. Mutation Research 275, 157-167

Aruoma OI, Halliwell B, Hoey BM, Butler J (1988): The antioxidant action of taurine, hypotaurine and their metabolic precursors. Biochemical Journal 256, 251-255

Astley SB, Elliott RM (2005): How strong is the evidence that lycopene supplementation can modify biomarkers of oxidative damage and DNA repair in human lymphocytes? Journal of Nutrition 135, 2071s-2073s

Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A, Sharma RK (2000): The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. International Journal of Fertility and Womens Medicine 45, 314-320

Bai J, Cederbaum AI (2001): Mitochondrial catalase and oxidative injury. Biological Signals and Receptors 10, 189-199

Bai J, Rodriguez AM, Melendez JA, Cederbaum AI (1999): Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury. The Journal of Biological Chemistry 274, 26217-26224

Bain NT, Madan P, Betts DH (2011): The early embryo response to intracellular reactive oxygen species is developmentally regulated. Reproduction Fertility and Development 23, 561-575

Bajt ML, Cover C, Lemasters JJ, Jaeschke H (2006): Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicological Sciences 94, 217-225

Baker MA, Aitken RJ (2005): Reactive oxygen species in spermatozoa: methods for monitoring and significance for the origins of genetic disease and infertility. Reproductive Biology and Endocrinology 3, 67

Balaban RS, Nemoto S, Finkel T (2005): Mitochondria, oxidants, and aging. Cell 120, 483-495

Balasubramanian S, Son WJ, Kumar BM, Ock SA, Yoo JG, Im GS, Choe SY, Rho GJ (2007): Expression pattern of oxygen and stress-responsive gene transcripts at various developmental stages of in vitro and in vivo preimplantation bovine embryos. Theriogenology 68, 265-275

Ballinger SW (2005): Mitochondrial dysfunction in cardiovascular disease. Free Radical Biology and Medicine 38, 1278-1295

Barcelo-Fimbres M, Seidel GE, Jr. (2007a): Effects of either glucose or fructose and metabolic regulators on bovine embryo development and lipid accumulation in vitro. Molecular Reproduction and Development 74, 1406-1418

Barcelo-Fimbres M, Seidel GE, Jr. (2007b): Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during in vitro culture of bovine embryos on embryonic development after cryopreservation. Molecular Reproduction and Development 74, 1395-1405

Barnett DK, Bavister BD (1992): Hypotaurine requirement for invitro development of golden-hamster one-cell embryos into morulae and blastocysts, and production of term offspring from invitro-fertilized Ova. Biology of Reproduction 47, 297-304

Barnett DK, Bavister BD (1996): What is the relationship between the metabolism of preimplantation embryos and their developmental competence? Molecular Reproduction and Development 43, 105-133

Barrenetxea G, de Larruzea AL, Ganzabal T, Jimenez R, Carbonero K, Mandiola M (2005): Blastocyst culture after repeated failure of cleavage-stage embryo transfers: a comparison of day 5 and day 6 transfers. Fertility and Sterility 83, 49-53

Bashan N, Kovsan J, Kachko I, Ovadia H, Rudich A (2009): Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. Physiological Reviews 89, 27-71

Basu S (2010): Fatty acid oxidation and isoprostanes: Oxidative strain and oxidative stress. Prostaglandins Leukotrienes and Essential Fatty Acids 82, 219-225

Bazer FW, Geisert RD, Zavy MT (1987): Fertilisation, cleavage and implantation. In Reproduction in Farm Animals. E.S.E. Hafez, editor. Lea and Febiger, Philadelphia pp 210-228

Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J, Agarwal A (2004): Differential growth of human embryos in vitro: role of reactive oxygen species. Fertility and Sterility 82, 593-600

Bedard K, Krause KH (2007): The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. Physiological Reviews 87, 245-313

Beehler BC, Przybyszewski J, Box HB, Kuleszmartin MF (1992): Formation of 8-Hydroxydeoxyguanosine within DNA of mouse keratinocytes exposed in culture to UVb and H_2O_2. Carcinogenesis 13, 2003-2007

Behboodi E, Anderson GB, Bondurant RH, Cargill SL, Kreuscher BR, Medrano JF, Murray JD (1995): Birth of large calves that developed from in vitro-derived bovine embryos. Theriogenology 44, 227-232

Ben-Dor A, Steiner M, Gheber L, Danilenko M, Dubi N, Linnewiel K, Zick A, Sharoni Y, Levy J (2005): Carotenoids activate the antioxidant response element transcription system. Molecular Cancer Therapeutics 4, 177-186

Berg JM, Tymoczko JL, L. S (2002): Biochemistry 5th Edition. New York: W H Freeman Section 18.13

Bernardi ML, Delouis C (1996): Sex-related differences in the developmental rate of in-vitro matured/in-vitro fertilized ovine embryos. Hum Reprod 11, 621-626

Betteridge DJ (2000): What is oxidative stress? Metabolism-Clinical and Experimental 49, 3-8

Betteridge KJ (2003): A history of farm animal embryo transfer and some associated techniques. Animal Reproduction Science 79, 203-244

Betteridge KJ, Flechon JE (1988): The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 29, 155-187

Bettger WJ (1993): Zinc and selenium, site-specific versus general antioxidation. Canadian Journal of Physiology and Pharmacology 71, 721-724

Bevilacqua E, Gomes SZ, Lorenzon AR, Hoshida MS, Amarante-Paffaro AM (2012): NADPH oxidase as an important source of reactive oxygen species at the mouse maternal-fetal interface: putative biological roles. Reproductive Biomedicine Online 25, 31-43

Bhandari D, Hossein M, Jeong YW, Kim S, Kim JH, Hwang WS (2007): Effects of glutathione on the in vitro development of porcine embryos in two different culture media. Biology of Reproduction 89-89

Bhandary B, Marahatta A, Kim HR, Chae HJ (2013): An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. International Journal of Molecular Sciences 14, 434-456

Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C (2000): Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Molecular Reproduction and Development 55, 282-288

Bing YZ, Hirao Y, Takenouchi N, Che LM, Nakamura H, Yodoi J, Nagai T (2003): Effects of thioredoxin on the preimplantation development of bovine embryos. Theriogenology 59, 863-873

Boldin MP, Goncharov TM, Goltsev YV, Wallach D (1996): Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85, 803-815

Bols PEJ, Goovaerts IGF, Jorssen EPA, Petro EML, Langbeen A, Leroy JLMR (2012): New applications for bovine IVP technology: from 'single oocyte culture' to toxicity screening. Anim Reprod 9, 388-394

Booth PJ, Holm P, Callesen H (2005): The effect of oxygen tension on porcine embryonic development is dependent on embryo type. Theriogenology 63, 2040-2052

Brevini TA, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F (2002): Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. Molecular Reproduction and Development 63, 510-517

Brown DM, Donaldson K, Stone V (2010): Nuclear translocation of Nrf2 and expression of antioxidant defence genes in THP-1 cells exposed to carbon nanotubes. Journal of Biomedical Nanotechnology 6, 224-233

Burton GJ, Jauniaux E (2011): Oxidative stress. Best Practice & Research Clinical Obstetrics & Gynaecology 25, 287-299

Burton GW, Foster DO, Perly B, Slater TF, Smith ICP, Ingold KU (1985): Biological antioxidants. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 311, 565-578

Buytaert E, Dewaele M, Agostinis P (2007): Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochimica Et Biophysica Acta-Reviews on Cancer 1776, 86-107

Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ, Camello PJ (2006): Mitochondrial reactive oxygen species and Ca^{2+} signaling. American Journal of Physiology-Cell Physiology 291, C1082-C1088

Carolan C, Lonergan P, Khatir H, Mermillod P (1996): In vitro production of bovine embryos using individual oocytes. Molecular Reproduction and Development 45, 145-150

Catt JW, Henman M (2000): Toxic effects of oxygen on human embryo development. Human Reproduction 15, 199-206

Cerutti P, Ghosh R, Oya Y, Amstad P (1994): The role of the cellular antioxidant defense in oxidant carcinogenesis. Environmental Health Perspectives 102, 123-129

Cerutti PA (1994): Oxyradicals and cancer. Lancet 344, 862-863

Cervinka M, Bereiter-Hahn J, Peychl J, Rudolf E, Cervinkova Z (1999): The role of mitochondria in apoptosis induced in vitro. General Physiology and Biophysics 18, 33-40

Cetica PD, Pintos LN, Dalvit GC, Beconi MT (2001): Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. IUBMB Life 51, 57-64

Cetica PD, Pintos LN, Dalvit GC, Beconi MT (1999): Effect of lactate dehydrogenase activity and isoenzyme localization in bovine oocytes and utilization of oxidative substrates on in vitro maturation. Theriogenology 51, 541-550

Chan KM, Lu RH, Chang JC, Kan YW (1996): NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proceedings of the National Academy of Sciences of the United States of America 93, 13943-13948

Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K, Yamamoto M, Hayes JD (2002): Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. Biochemical Journal 365, 405-416

Chandel NS, Budinger GRS (2007): The cellular basis for diverse responses to oxygen. Free Radical Biology and Medicine 42, 165-174

Chandra A, Surti N, Kesavan S, Agarwal A (2009): Significance of oxidative stress in human reproduction. Archives of Medical Science 5, S28-S42

Chao CC, Ma YS, Stadtman ER (1997): Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. Proceedings of the National Academy of Sciences of the United States of America 94, 2969-2974

Chappel S (2013): The role of mitochondria from mature oocyte to viable blastocyst. Obstetrics and Gynecology International 2013, 1-10

Chaube SK, Prasad PV, Khillare B, Shrivastav TG (2006): Extract of Azadirachta indica (neem) leaf induces apoptosis in rat oocytes cultured in vitro. Fertility and Sterility 85, 1223-1231

Chaudhari N, Talwar P, Parimisetty A, d'Hellencourt CL, Ravanan P (2014): A molecular web: endoplasmic reticulum stress, inflammation, and oxidative stress. Frontiers in Cellular Neuroscience 8, 213

Cheeseman KH (1993): Mechanisms and effects of lipid-peroxidation. Molecular Aspects of Medicine 14, 191-197

Chen B, Zhang YZ, Wang Y, Rao J, Jiang XM, Xu ZH (2014): Curcumin inhibits proliferation of breast cancer cells through Nrf2-mediated down-regulation of Fen1 expression. Journal of Steroid Biochemistry and Molecular Biology 143, 11-18

Chen H, Li JY, Li HY, Hu YH, Tevebaugh W, Yamamoto M, Que JW, Chen XX (2012): Transcript profiling identifies dynamic gene expression patterns and an important role for Nrf2/Keap1 pathway in the developing mouse esophagus. Plos One 7,

Chen XL, Dodd G, Thomas S, Zhang XL, Wasserman MA, Rovin BH, Kunsch C (2006): Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. American Journal of Physiology-Heart and Circulatory Physiology 290, H1862-H1870

Chen XL, Kunsch C (2004): Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: A new therapeutic approach for the treatment of inflammatory diseases. Current Pharmaceutical Design 10, 879-891

Cheung KL, Lee JH, Khor TO, Wu TY, Li GX, Chan J, Yang CS, Kong AN (2012): Nrf2 knockout enhances intestinal tumorigenesis in Apc(min/+) mice due to attenuation of anti-oxidative stress pathway while potentiates inflammation. Molecular Carcinogenesis 53, 77-84

Chinnaiyan AM, Tepper CG, Seldin MF, ORourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME, Dixit VM (1996): FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. The Journal of Biological Chemistry 271, 4961-4965

Chipuk JE, Green DR (2008): How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends in Cell Biology 18, 157-164

Choi SH, Cho SR, Han MH, Kim HJ, Choe CY, Son DS, Kim YK, Chung YG, Hoshi H (2006): Effects of apotransferrin on in vitro maturation of cumulus-oocytes complexes (COCs) in Hanwoo, Korean native cows. Reproduction Fertility and Development 18, 187-187

Cindrova-Davies T, Yung HW, Johns J, Spasic-Boskovic O, Korolchuk S, Jauniaux E, Burton GJ, Charnock-Jones DS (2007): Oxidative stress, gene expression, and protein changes induced in the human placenta during labor. American Journal of Pathology 171, 1168-1179

Cohen J, Gilligan A, Esposito W, Schimmel T, Dale B (1997): Ambient air and its potential effects on conception in vitro. Human Reproduction 12, 1742-1749

Cooke MS, Evans MD, Dizdaroglu M, Lunec J (2003): Oxidative DNA damage: mechanisms, mutation, and disease. Faseb J 17, 1195-1214

Corcoran D, Fair T, Park S, Rizos D, Patel OV, Smith GW, Coussens PM, Ireland JJ, Boland MP, Evans AC, Lonergan P (2006): Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos. Reproduction 131, 651-660

Correa GA, Rumpf R, Mundim TCD, Franco MM, Dode MAN (2008): Oxygen tension during in vitro culture of bovine embryos: effect in production and expression of genes related to oxidative stress. Animal Reproduction Science 104, 132-142

Covarrubias L, Hernandez-Garcia D, Schnabel D, Salas-Vidal E, Castro-Obregon S (2008): Function of reactive oxygen species during animal development: Passive or active? Developmental Biology 320, 1-11

Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE (2000): Ultrastructural morphometry of bovine compact morulae produced in vivo or in vitro. Biology of Reproduction 62, 1459-1465

Cui H, Kong Y, Zhang H (2012): Oxidative stress, mitochondrial dysfunction, and aging. Journal of Signal Transduction 2012, 646354

Curnow EC, Ryan JP, Saunders DM, Hayes ES (2010): Developmental potential of bovine oocytes following IVM in the presence of glutathione ethyl ester. Reproduction Fertility and Development 22, 597-605

Curnow EC, Ryan JP, Saunders DM, Hayes ES (2011): Primate model of metaphase I oocyte in vitro maturation and the effects of a novel glutathione donor on maturation, fertilization, and blastocyst development. Fertility and Sterility 95, 1235-1240

Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A (2006): Biomarkers of oxidative damage in human disease. Clinical Chemistry 52, 601-623

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003): Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329, 23-38

Dalvit G, Llanes SP, Descalzo A, Insani M, Beconi M, Cetica P (2005): Effect of alpha-tocopherol and ascorbic acid on bovine oocyte in vitro maturation. Reproduction in Domestic Animals 40, 93-97

Dalzell LH, McVicar CM, McClure N, Lutton D, Lewis SEM (2004): Effects of short and long incubations on DNA fragmentation of testicular sperm. Fertility and Sterility 82, 1443-1445

Das S, Chattopadhyay R, Ghosh S, Goswami SK, Chakravarty BN, Chaudhury K (2006): Reactive oxygen species level in follicular fluid--embryo quality marker in IVF? Human Reproduction 21, 2403-2407

Dashti SI, Thomson M, Mameesh MS (1995): Effects of copper deficiency and Cu complexes on superoxide dismutase in rats. Nutrition 11, 564-567

Davies KJA (2000): An overview of oxidative stress. IUBMB Life 50, 241-244

Davies MJ (2005): The oxidative environment and protein damage. Biochimica Et Biophysica Acta-Proteins and Proteomics 1703, 93-109

Davies MJ, Fu SL, Dean RT (1995): Protein hydroperoxides can give rise to reactive free-radicals. Biochemical Journal 305, 643-649

De AK, Malakar D, Akshey YS, Jena MK, Garg S, Dutta R, Sahu S (2011): In vitro development of goat (Capra hircus) embryos following cysteamine supplementation of the in vitro maturation and in vitro culture media. Small Ruminant Research 96, 185-190

De Bont R, van Larebeke N (2004): Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19, 169-185

de Matos DG, Furnus CC (2000): The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: Effect of beta-mercaptoethanol, cysteine and cystine. Theriogenology 53, 761-771

de Matos DG, Furnus CC, Moses DF, Martinez AG, Matkovic M (1996): Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. Molecular Reproduction and Development 45, 451-457

de Matos DG, Herrera C, Cortvrindt R, Smitz J, Van Soom A, Nogueira D, Pasqualini RS (2002): Cysteamine supplementation during in vitro maturation and embryo culture: A useful tool for increasing the efficiency of bovine in vitro embryo production. Molecular Reproduction and Development 62, 203-209

De Vizcaya-Ruiz A, Barbier O, Ruiz-Ramos R, Cebrian ME (2009): Biomarkers of oxidative stress and damage in human populations exposed to arsenic. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 674, 85-92

Dean RT, Fu SL, Stocker R, Davies MJ (1997): Biochemistry and pathology of radical-mediated protein oxidation. Biochemical Journal 324, 1-18

Dean RT, Thomas SM, Vince G, Wolff SP (1986): Oxidation induced proteolysis and its possible restriction by some secondary protein modifications. Biomedica Biochimica Acta 45, 1563-1573

Dematos DG, Furnus CC, Moses DF, Baldassarre H (1995): Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in-vitro. Molecular Reproduction and Development 42, 432-436

Dhakshinamoorthy S, Jaiswal AK (2001): Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. Oncogene (London) 20, 3906-3917

Dix TA, Aikens J (1993): Mechanisms and biological relevance of lipid-peroxidation initiation. Chemical Research in Toxicology 6, 2-18

Dizdaroglu M (1992): Oxidative damage to DNA in mammalian chromatin. Mutation Research 275, 331-342

Dizdaroglu M (1993): Quantitative-Determination of oxidative base damage in DNA by stable isotope-dilution mass-spectrometry. Febs Lett 315, 1-6

Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H (2002): Free radical-induced damage to DNA: Mechanisms and measurement. Free Radical Biology and Medicine 32, 1102-1115

Dizdaroglu M, Olinski R, Doroshow JH, Akman SA (1993): Modification of DNA bases in chromatin of intact target human-cells by activated human polymorphonuclear leukocytes. Cancer Res 53, 1269-1272

Dode MA, Dufort I, Massicotte L, Sirard MA (2006): Quantitative expression of candidate genes for developmental competence in bovine two-cell embryos. Molecular Reproduction and Development 73, 288-297

Doherty AS, Mann MRW, Tremblay KD, Bartolomei MS, Schultz RM (2000): Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biology of Reproduction 62, 1526-1535

Dong J, Sulik KK, Chen SY (2008): Nrf2-mediated transcriptional induction of antioxidant response in mouse embryos exposed to ethanol in vivo: implications for the prevention of fetal alcohol spectrum disorders. Antioxidants & Redox Signaling 10, 2023-2033

Downs SM, Dow MPD (1991): Hypoxanthine-Maintained 2-cell block in mouse embryos dependence on glucose and effect of hypoxanthine phosphoribosyltransferase inhibitors. Biology of Reproduction 44, 1025-1039

Downs SM, Mastropolo AM (1994): The participation of energy substrates in the control of meiotic maturation in murine oocytes. Developmental Biology 162, 154-168

Dreger H, Westphal K, Weller A, Baumann G, Stangl V, Meiners S, Stangl K (2009): Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for proteasome inhibitor-mediated cardioprotection. Cardiovascular Research 83, 354-361

Droge W (2002): Free radicals in the physiological control of cell function. Physiological Reviews 82, 47-95

Du ZF, Wales RG (1993): Glycolysis and glucose oxidation by the sheep conceptus at different oxygen concentrations. Reproduction Fertility and Development 5, 383-393

Dumoulin JC, Meijers CJ, Bras M, Coonen E, Geraedts JP, Evers JL (1999): Effect of oxygen concentration on human in-vitro fertilization and embryo culture. Human Reproduction 14, 465-469

Dumoulin JCM, Evers JLH, Bras M, Pieters MHEC, Geraedts JPM (1992): Positive effect of taurine on preimplantation development of mouse embryos invitro. Journal of Reproduction and Fertility 94, 373-380

Duru NK, Morshedi M, Oehninger S (2000): Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertility and Sterility 74, 1200-1207

Eberle D, Hegarty B, Bossard P, Ferre P, Foufelle F (2004): SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 86, 839-848

El Mouatassim S, Guerin P, Menezo Y (1999): Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. Molecular Human Reproduction 5, 720-725

El Shourbagy SH, Spikings EC, Freitas M, St John JC (2006): Mitochondria directly influence fertilisation outcome in the pig. Reproduction 131, 233-245

Elamaran G, Singh KP, Singh MK, Singla SK, Chauhan MS, Manik RS, Palta P (2012): Oxygen concentration and cysteamine supplementation during in vitro production of Buffalo (Bubalus bubalis) embryos affect mRNA expression of BCL-2, BCL-XL, MCL-1, BAX and BID. Reproduction in Domestic Animals 47, 1027-1036

Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T, Yamamoto M (2001): High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicological Sciences 59, 169-177

Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP (2000): Culture of in vitro produced bovine zygotes in vitro vs in vivo: Implications for early embryo development and quality. Theriogenology 54, 659-673

Epe B, Pflaum M, Boiteux S (1993): DNA damage induced by photosensitizers in cellular and cell-free systems. Mutation Research 299, 135-145

Exley GE, Tang CY, McElhinny AS, Warner CM (1999): Expression of caspase and BCL-2 apoptotic family members in mouse preimplantation embryos. Biology of Reproduction 61, 231-239

Fair T, Murphy M, Rizos D, Moss C, Martin F, Boland MP, Lonergan P (2004): Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. Molecular Reproduction and Development 67, 136-144

Falkenberg M, Larsson NG, Gustafsson CM (2007): DNA replication and transcription in mammalian mitochondria. Annual Review of Biochemistry 76, 679-699

Fan J, Cai H, Tan WS (2007): Role of the plasma membrane ROS-generating NADPH oxidase in CD34(+) progenitor cells preservation by hypoxia. Journal of Biotechnology 130, 455-462

Farin PW, Crosier AE, Farin CE (2001): Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 55, 151-170

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

Farin PW, Piedrahita JA, Farin CE (2006): Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 65, 178-191

Favetta LA, St John EJ, King WA, Betts DH (2007): High levels of p66shc and intracellular ROS in permanently arrested early embryos. Free Radical Biology & Medicine 42, 1201-1210

Ferguson EM, Leese HJ (1999): Triglyceride content of bovine oocytes and early embryos. Journal of Reproduction and Fertility 116, 373-378

Ferre P, Foufelle F (2007): SREBP-1c transcription factor and lipid homeostasis: clinical perspective. Hormone Research 68, 72-82

Ferri KF, Kroemer G (2001): Organelle-Specific initiation of cell death pathways. Nature Cell Biology 3, E255-E263

Feugang JM, De Roover R, Moens A, Leonard S, Dessy F, Donnay I (2004): Addition of beta-mercaptoethanol or Trolox (R) at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. Theriogenology 61, 71-90

Fingerova H, Oborna I, Novotny J, Svobodova M, Brezinova J, Radova L (2009): The measurement of reactive oxygen species in human neat semen and in suspended spermatozoa: a comparison. Reproductive Biology and Endocrinology 7, 118

Finkel T (2011): Signal transduction by reactive oxygen species. Journal of Cell Biology 194, 7-15

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

Fleming JE, Miquel J, Cottrell SF, Yengoyan LS, Economos AC (1982): Is cell aging caused by respiration-dependent injury to the mitochondrial genome. Gerontology 28, 44-53

Foote CS (1991): Definition of Type-I and Type-Ii Photosensitized Oxidation. Photochemistry and Photobiology 54, 659-659

Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN (1991): Ascorbic-Acid protects against endogenous oxidative DNA damage in human sperm. Proceedings of the National Academy of Sciences of the United States of America 88, 11003-11006

Frand AR, Kaiser CA (1999): Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. Molecular Cell 4, 469-477

Frandsen A, Schousboe A (1993): Excitatory amino acid-mediated cytotoxicity and calcium homeostasis in cultured neurons. Journal of Neurochemistry 60, 1202-1211

Fridovich SE, Porter NA (1981): Oxidation of arachidonic-acid in micelles by superoxide and hydrogen-peroxide. The Journal of Biological Chemistry 256, 260-265

Frostesjo L, Holm I, Grahn B, Page AW, Bestor TH, Heby O (1997): Interference with DNA methyltransferase activity and genome methylation during F9

teratocarcinoma stem cell differentiation induced by polyamine depletion. The Journal of Biological Chemistry 272, 4359-4366

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

Fujitani Y, Kasai K, Ohtani S, Nishimura K, Yamada M, Utsumi K (1997): Effect of oxygen concentration and free radicals on in vitro development of in vitro-produced bovine embryos. Journal of Animal Science 75, 483-489

Fukuhara R, Fujii S, Nakamura R, Yuzawa E, Kimura H, Fukui A, Mizunuma H (2008): Erythrocytes counteract the negative effects of female ageing on mouse preimplantation embryo development and blastocyst formation. Human Reproduction 23, 2080-2085

Fukui Y, McGowan LT, James RW, Pugh PA, Tervit HR, (1991): Factors affecting the in-vitro development to blastocysts of bovine oocytes matured and fertilized in vitro. Reproduction 92, 125-131

Fulka J, Moor RM, Fulka J (1995): Mouse oocyte maturation - meiotic checkpoints. Experimental Cell Research 219, 414-419

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

Gardiner CS, Reed DJ (1994): Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. Biology of Reproduction 51, 1307-1314

Gardiner CS, Reed DJ (1995): Synthesis of glutathione in the preimplantation mouse embryo. Archives of Biochemistry and Biophysics 318, 30-36

Gardiner CS, Salmen JJ, Brandt CJ, Stover SK (1998): Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically induced glutathione depletion. Biology of Reproduction 59, 431-436

Gardner DK, Hamilton R, McCallie B, Schoolcraft WB, Katz-Jaffe MG (2013): Human and mouse embryonic development, metabolism and gene expression are altered by an ammonium gradient in vitro. Reproduction 146, 49-61

Gavella M, Lipovac V (1992): Nadh-Dependent oxidoreductase (Diaphorase) activity and isozyme pattern of sperm in infertile men. Archives of Andrology 28, 135-141

Ge HS, Tollner TL, Hu Z, Dai MM, Li XH, Guan HQ, Shan D, Zhang XJ, Lv JQ, Huang CJ, Dong QX (2012): The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. Molecular Reproduction and Development 79, 392-401

Ghanem N, Holker M, Rings F, Jennen D, Tholen E, Sirard MA, Torner H, Kanitz W, Schellander K, Tesfaye D (2007): Alterations in transcript abundance of bovine oocytes recovered at growth and dominance phases of the first follicular wave. BMC Developmental Biology 7, 90

Gill SS, Tuteja N (2010): Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48, 909-930

Girotti AW (1998): Lipid hydroperoxide generation, turnover, and effector action in biological systems. Journal of Lipid Research 39, 1529-1542

Girotti AW (2001): Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. Journal of Photochemistry and Photobiology B-Biology 63, 103-113

Giudice A, Arra C, Turco MC (2010): Review of molecular mechanisms involved in the activation of the Nrf2-ARE signaling pathway by chemopreventive agents. Methods in Molecular Biology (Clifton, N.J.) 647, 37-74

Gong P, Cederbaum AI (2006a): Nrf2 is increased by CYP2E1 in rodent liver and HepG2 cells and protects against oxidative stress caused by CYP2E1. Hepatology (St. Louis, MO) 43, 144-153

Gong P, Cederbaum AI (2006b): Transcription factor Nrf2 protects HepG2 cells against CYP2E1 plus arachidonic acid-dependent toxicity. The Journal of Biological Chemistry 281, 14573-14579

Goossens K, Van Poucke M, Van Soom A, Vandesompele J, Van Zeveren A, Peelman LJ (2005): Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Developmental Biology 5, 27

Goossens K, Vandaele L, Wydooghe E, Thys M, Dewulf J, Peelman LJ, Van Soom A (2011): The importance of adequate fixation for immunofluorescent staining of bovine embryos. Reproduction in Domestic Animals 46, 1098-1103

Gorrini C, Harris IS, Mak TW (2013): Modulation of oxidative stress as an anticancer strategy. Nature Reviews Drug Discovery 12, 931-947

Goto Y, Noda Y, Mori T, Nakano M (1993): Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radical Biology & Medicine 15, 69-75

Goto Y, Noda Y, Narimoto K, Umaoka Y, Mori T (1992): Oxidative stress on mouse embryo development invitro. Free Radical Biology & Medicine 13, 47-53

Goud AP, Goud PT, Diamond MP, Gonik B, Abu-Soud HM (2008): Reactive oxygen species and oocyte aging: Role of superoxide, hydrogen peroxide, and hypochlorous acid. Free Radical Biology & Medicine 44, 1295-1304

Grant AJ, Jessup W, Dean RT (1993): Enhanced enzymatic degradation of radical damaged mitochondrial-membrane components. Free Radical Research Communications 19, 125-134

Graziewicz MA, Day BJ, Copeland WC (2002): The mitochondrial DNA polymerase as a target of oxidative damage. Nucleic Acids Research 30, 2817-2824

Greco T, Fiskum G (2010): Brain mitochondria from rats treated with sulforaphane are resistant to redox-regulated permeability transition. Journal of Bioenergetics and Biomembranes 42, 491-497

Griendling KK, Sorescu D, Ushio-Fukai M (2000): NAD(P)H oxidase - Role in cardiovascular biology and disease. Circulation Research 86, 494-501

Grisart B, Massip A, Dessy F (1994): Cinematographic analysis of bovine embryo development in serum-free oviduct-conditioned medium. Journal of Reproduction and Fertility 101, 257-264

Grupen CG, Nagashima H, Nottle MB (1995): Cysteamine enhances in-vitro development of porcine oocytes matured and fertilized in-vitro. Biology of Reproduction 53, 173-178

Guerin P, El Mouatassim S, Menezo Y (2001): Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Human Reproduction Update 7, 175-189

Gupta S, Malhotra N, Sharma D, Chandra A, Ashok A (2009): Oxidative stress and its role in female infertility and assisted reproduction: clinical implications. International Journal of Fertility & Sterility 2, 147-164

Gutierrez-Adan A, Lonergan P, Rizos D, Ward FA, Boland MP, Pintado B, de la Fuente J (2001): Effect of the in vitro culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology 55, 1117-1126

Gutteridge JMC (1995): Lipid-Peroxidation and antioxidants as biomarkers of tissue-damage. Clinical Chemistry 41, 1819-1828

Guyader-Joly C, Ponchon S, Heyman Y, Menezo Y, Renard JP (1998): Effect of hypotaurine on development of in vitro-derived bovine embryos. Theriogenology 49, 201-201

Haddad JJ (2004): On the antioxidant mechanisms of Bcl-2: a retrospective of NF-kappa B signaling and oxidative stress. Biochem Bioph Res Co 322, 355-363

Halliwell B (1990): How to characterize a biological antioxidant. Free Radical Research Communications 9, 1-32

Hansen JM (2012): Thioredoxin redox status assessment during embryonic development: the redox Western. Methods in Molecular Biology (Clifton, N.J.) 889, 305-313

Hardy K, Wright CS, Franks S, Winston RML (2000): In vitro maturation of oocytes. British Medical Bulletin 56, 588-602

Harris C, Hansen JM (2012): Nrf2-Mediated resistance to oxidant-induced redox disruption in embryos. Birth Defects Research Part B-Developmental and Reproductive Toxicology 95, 213-218

Harris MH, Thompson CB (2000): The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death and Differentiation 7, 1182-1191

Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG (2004): Oxygen-regulated gene expression in bovine blastocysts. Biology of Reproduction 71, 1108-1119

Harvey AJ, Kind KL, Thompson JG (2002): REDOX regulation of early embryo development. Reproduction 123, 479-486

Harvey AJ, Navarrete Santos A, Kirstein M, Kind KL, Fischer B, Thompson JG (2007): Differential expression of oxygen-regulated genes in bovine blastocysts. Molecular Reproduction and Development 74, 290-299

Harvey MB, Arcellana-Panlilio MY, Zhang X, Schultz GA, Watson AJ (1995): Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo coculture. Biology of Reproduction 53, 532-540

Hasler JF (2000): In-vitro production of cattle embryos: problems with pregnancies and parturition. Human Reproduction 15, 47-58

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B (1995): Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 43, 141-152

Hawkins CL, Pattison DI, Davies MJ (2003): Hypochlorite-induced oxidation of amino acids, peptides and proteins. Amino Acids 25, 259-274

Hawkins CL, Rees MD, Davies MJ (2002): Superoxide radicals can act synergistically with hypochlorite to induce damage to proteins. Febs Lett 510, 41-44

He X, Lin GX, Chen MG, Zhang JX, Ma Q (2007): Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association. Toxicological Sciences 98, 298-309

Heby O (1995): DNA methylation and polyamines in embryonic development and cancer. International Journal of Developmental Biology 39, 737-757

Hegler J, Bittner D, Boiteux S, Epe B (1993): Quantification of oxidative DNA modifications in mitochondria. Carcinogenesis 14, 2309-2312

Held E, Salilew-Wondim D, Linke M, Zechner U, Rings F, Tesfaye D, Schellander K, Hoelker M (2012): Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere. Biology of Reproduction 87, 154

Henderson BW, Dougherty TJ (1992): How does photodynamic therapy work. Photochemistry and Photobiology 55, 145-157

Hendriks WK, Roelen BAJ, Colenbrander B, Stout TAE (2014): Cellular damage suffered by equine embryos after exposure to cryoprotectants or cryopreservation by slow-freezing or vitrification. . Equine Vet J. doi: 10.1111/evj.12341,

Hengartner MO (2000): The biochemistry of apoptosis. Nature 407, 770-776

Hiona A, Leeuwenburgh C (2008): The role of mitochondrial DNA mutations in aging and sarcopenia: Implications for the mitochondrial vicious cycle theory of aging. Experimental Gerontology 43, 24-33

Ho YS, Lee HM, Mou TC, Wang YJ, Lin JK (1997): Suppression of nitric oxide-induced apoptosis by N-acetyl-L-cysteine through modulation of glutathione, bcl-2, and bax protein levels. Mol Carcinogen 19, 101-113

Hockberger PE, Skimina TA, Centonze VE, Lavin C, Chu S, Dadras S, Reddy JK, White JG (1999): Activation of flavin-containing oxidases underlies light-induced production of H_2O_2 in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 96, 6255-6260

Holm P, Callesen H (1998): In vivo versus in vitro produced bovine ova: similarities and differences relevant for practical application. Reproduction Nutrition Development 38, 579-594

Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H (1998): Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. Theriogenology 50, 1285-1299

Honda Y, Tanikawa H, Fukuda J, Kawamura K, Sato N, Sato T, Shimizu Y, Kodama H, Tanaka T (2005): Expression of Smac/DIABLO in mouse preimplantation embryos and its correlation to apoptosis and fragmentation. Molecular Human Reproduction 11, 183-188

Hossein MS, Hashem MA, Jeong YW, Lee MS, Kim S, Kim JH, Koo OJ, Park SM, Lee EG, Park SW, Kang SK, Lee BC, Hwang WS (2007): Temporal effects of alpha-tocopherol and L-ascorbic acid on in vitro fertilized porcine embryo development. Animal Reproduction Science 100, 107-117

Houghton FD, Thompson JG, Kennedy CJ, Leese HJ (1996): Oxygen consumption and energy metabolism of the early mouse embryo. Molecular Reproduction and Development 44, 476-485

Hu J, Cheng D, Gao X, Bao J, Ma X, Wang H (2012): Vitamin C enhances the in vitro development of porcine pre-implantation embryos by reducing oxidative stress. Reproduction in Domestic Animals 47, 873-879

Huang J, Tabbi-Anneni I, Gunda V, Wang L (2010): Transcription factor Nrf2 regulates SHP and lipogenic gene expression in hepatic lipid metabolism. American journal of Physiology. Gastrointestinal and liver physiology (Bethesda, MD) 299, G1211-1221

Huang YY, Tang XC, Xie WH, Zhou Y, Li D, Zhou Y, Zhu JG, Yuan T, Lai LX, Pang DX, Ouyang HS (2011): Vitamin C enhances in vitro and in vivo development of porcine somatic cell nuclear transfer embryos. Biochem Bioph Res Co 411, 397-401

Hunter AM, LaCasse EC, Korneluk RG (2007): The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis 12, 1543-1568

Huttemann M, Lee I, Samavati L, Yu H, Doan JW (2007): Regulation of mitochondrial oxidative phosphorylation through cell signaling. Biochimica Et Biophysica Acta-Molecular Cell Research 1773, 1701-1720

Hyttel P, Viuff D, Laurincik J, Schmidt M, Thomsen PD, Avery B, Callesen H, Rath D, Niemann H, Rosenkranz C, Schellander K, Ochs RL, Greve T (2000): Risks of in-vitro production of cattle and swine embryos: aberrations in chromosome numbers, ribosomal RNA gene activation and perinatal physiology. Human Reproduction 15, 87-97

Im JY, Lee KW, Woo JM, Junn E, Mouradian MM (2012): DJ-1 induces thioredoxin 1 expression through the Nrf2 pathway. Human Molecular Genetics 21, 3013-3024

Ishizuka B, Kuribayashi Y, Murai L, Amemiya A, Itoh MT (2000): The effect of melatonin on in vitro fertilization and embryo development in mice. Journal of Pineal Research 28, 48-51

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997): An Nrf2 small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Bioph Res Co 236, 313-322

Itoh K, Igarashi K, Hayashi N, Nishizawa M, Yamamoto M (1995): Cloning and characterization of a novel erythroid cell-derived cnc family transcription

factor heterodimerizing with the small Maf family proteins. Molecular and Cellular Biology (Washington, DC) 15, 4184-4193

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

Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T, Yamamoto M (2003): Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. Genes to Cells 8, 379-391

Iudica C, Kaiser G, Alberio R (1999): Antioxidants during in vitro oocyte maturation (IVM) affect the in vitro production (IVP) of bovine embryos. Theriogenology 51, 378-378

Iwasaki A, Gagnon C (1992): Formation of reactive oxygen species in spermatozoa of infertile patients. Fertility and Sterility 57, 409-416

Iwata H, Akamatsu S, Minami N, Yamada M (1998): Effects of antioxidants on the development of bovine IVM/IVF embryos in various concentrations of glucose. Theriogenology 50, 365-375

Jena NR (2012): DNA damage by reactive species: Mechanisms, mutation and repair. Journal of Biosciences 37, 503-517

Jena NR, Mishra PC (2005): Mechanisms of formation of 8-oxoguanine due to reactions of one and two OH center dot radicals and the H_2O_2 molecule with guanine: A quantum computational study. Journal of Physical Chemistry B 109, 14205-14218

Jeong SY, Seol DW (2008): The role of mitochondria in apoptosis. Bmb Reports 41, 11-22

Joenje H (1987): Cellular defense-mechanisms in free-radical toxicology. Pharmaceutisch Weekblad-Scientific Edition 9, 36-36

Johnson MH, Nasr-Esfahani MH (1994): Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? Bioessays 16, 31-38

Jungas T, Motta I, Duffieux F, Fanen P, Stoven V, Ojcius DM (2002): Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells

expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. The Journal of Biological Chemistry 277, 27912-27918

Jurisicova A, Acton BM (2004): Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. Reproduction 128, 281-291

Jurisicova A, Antenos M, Varmuza S, Tilly JL, Casper RF (2003): Expression of apoptosis-related genes during human preimplantation embryo development: potential roles for the Harakiri gene product and Caspase-3 in blastomere fragmentation. Molecular Human Reproduction 9, 133-141

Jurisicova A, Latham KE, Casper RF, Varmuza SL (1998): Expression and regulation of genes associated with cell death during murine preimplantation embryo development. Molecular Reproduction and Development 51, 243-253

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

Kalthur G, Salian SR, Keyvanifard F, Sreedharan S, Thomas JS, Kumar P, Adiga SK (2012): Supplementation of biotin to sperm preparation medium increases the motility and longevity in cryopreserved human spermatozoa. Journal of Assisted Reproduction and Genetics 29, 631-635

Kamiya H (2003): Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides. Nucleic Acids Research 31, 517-531

Karja NWK, Wongsrikeao P, Murakami M, Agung B, Fahrudin M, Nagai T, Otoi T (2004): Effects of oxygen tension on the development and quality of porcine in vitro fertilized embryos. Theriogenology 62, 1585-1595

Kasahara Y, Iwai K, Yachie A, Ohta K, Konno A, Seki H, Miyawaki T, Taniguchi N (1997): Involvement of reactive oxygen intermediates in spontaneous and CD95(Fas/APO-1)-mediated apoptosis of neutrophils. Blood 89, 1748-1753

Katayama M, Rieke A, Cantley T, Murphy C, Dowell L, Sutovsky P, Day BN (2007): Improved fertilization and embryo development resulting in birth of live piglets after intracytoplasmic sperm injection and in vitro culture in a cysteine-supplemented medium. Theriogenology 67, 835-847

Kawamura K, Fukuda J, Kodama H, Kumagai J, Kumagai A, Tanaka T (2001): Expression of Fas and Fas ligand mRNA in rat and human preimplantation embryos. Molecular Human Reproduction 7, 431-436

Kawamura Y, Uchijima Y, Horike N, Tonami K, Nishiyama K, Amano T, Asano T, Kurihara Y, Kurihara H (2010): Sirt3 protects in vitro-fertilized mouse preimplantation embryos against oxidative stress-induced p53-mediated developmental arrest. Journal of Clinical Investigation 120, 2817-2828

Kehrer JP (2000): The Haber-Weiss reaction and mechanisms of toxicity. Toxicology 149, 43-50

Kelekar A, Thompson CB (1998): Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends in Cell Biology 8, 324-330

Khor TO, Huang MT, Prawan A, Liu Y, Hao X, Yu S, Cheung WK, Chan JY, Reddy BS, Yang CS, Kong AN (2008): Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. Cancer Prevention Research 1, 187-191

Khoudja RY, Xu YW, Li T, Zhou CQ (2013): Better IVF outcomes following improvements in laboratory air quality. Journal of Assisted Reproduction and Genetics 30, 69-76

Khurana NK, Niemann H (2000): Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. Biology of Reproduction 62, 847-856

Khurana NK, Wales RG (1989): Effects of oxygen concentration on the metabolism of [U-14C]glucose by mouse morulae and early blastocysts in vitro. Reprod Fertil Dev 1, 99-106

Kim HJ, Vaziri ND (2009): Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure. American Journal of Physiology. Gastrointestinal and Liver Physiology (Bethesda, MD) 298, F662-671

Kim JH, Lee SH, Kim S, Jeong YW, Koo OJ, Hashem MDA, Park SM, Lee EG, Hossein MS, Kang SK, Lee BC, Hwang WS (2006): Embryotrophic effects of ethylenediaminetetraacetic acid and hemoglobin on in vitro porcine embryos development. Theriogenology 66, 449-455

Kim S, Agca C, Agca Y (2013): Effects of various physical stress factors on mitochondrial function and reactive oxygen species in rat spermatozoa. Reproduction Fertility and Development 25, 1051-1064

Kim YJ, Ahn JY, Liang P, Ip C, Zhang Y, Park YM (2007): Human prx1 gene is a target of Nrf2 and is up-regulated by hypoxia/reoxygenation: implication to tumor biology. Cancer Res 67, 546-554

Kimura N, Tsunoda S, Iuchi Y, Abe H, Totsukawa K, Fujii J (2010): Intrinsic oxidative stress causes either 2-cell arrest or cell death depending on developmental stage of the embryos from SOD1-deficient mice. Molecular Human Reproduction 16, 441-451

Kind KL, Collett RA, Harvey AJ, Thompson JG (2005): Oxygen-regulated expression of GLUT-1, GLUT-3, and VEGF in the mouse blastocyst. Molecular Reproduction and Development 70, 37-44

Kirkinezos IG, Moraes CT (2001): Reactive oxygen species and mitochondrial diseases. Seminars in Cell & Developmental Biology 12, 449-457

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME (1995): Cytotoxicity-Dependent Apo-1 (Fas/Cd95)-Associated proteins form a death-inducing signaling complex (Disc) with the receptor. Embo Journal 14, 5579-5588

Kitagawa Y, Suzuki K, Yoneda A, Watanabe T (2004): Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology 62, 1186-1197

Knijn HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Zeinstra EC, van der Weijden GC, Niemann H, Dieleman SJ (2005): In vitro and in vivo culture effects on mRNA expression of genes involved in metabolism and apoptosis in bovine embryos. Reproduction Fertility and Development 17, 775-784

Knorr-Wittmann C, Hengstermann A, Gebel S, Alam J, Muller T (2005): Characterization of Nrf2 activation and heme oxygenase-1 expression in NIH3T3 cells exposed to aqueous extracts of cigarette smoke. Free Radical Biology & Medicine 39, 1438-1448

Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, Yamamoto M (2006): Oxidative and electrophilic stresses activate Nrf2 through inhibition of

ubiquitination activity of Keap1. Molecular and Cellular Biology (Washington, DC) 26, 221-229

Kobayashi M, Itoh K, Suzuki T, Osanai H, Nishikawa K, Katoh Y, Takagi Y, Yamamoto M (2002): Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. Genes to Cells 7, 807-820

Kobayashi M, Yamamoto M (2005): Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. Antioxidants & Redox Signaling 7, 385-394

Koerber S, Santos AN, Tetens F, Kuchenhoff A, Fischer B (1998): Increased expression of NADH-ubiquinone oxidoreductase chain 2 (ND2) in preimplantation rabbit embryos cultured with 20% oxygen concentration. Mol Reprod Dev 49, 394-399

Kohli M, Yu J, Seaman C, Bardelli A, Kinzler KW, Vogelstein B, Lengauer C, Zhang L (2004): SMAC/Diablo-dependent apoptosis induced by nonsteroidal anti inflammatory drugs (NSAIDs) in colon cancer cells. Proceedings of the National Academy of Sciences of the United States of America 101, 16897-16902

Konstantinopoulos PA, Spentzos D, Fountzilas E, Francoeur N, Sanisetty S, Grammatikos AP, Hecht JL, Cannistra SA (2011): Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer. Cancer Res 71, 5081-5089

Kowaltowski AJ, Castilho RF, Vercesi AE (1995): $Ca^{(2+)}$-induced mitochondrial membrane permeabilization: role of coenzyme Q redox state. Am J Physiol 269, 141–147

Kowaltowski AJ, Vercesi AE (1999): Mitochondrial damage induced by conditions of oxidative stress. Free Radical Biology & Medicine 26, 463-471

Kroemer G, Galluzzi L, Brenner C (2007): Mitochondrial membrane permeabilization in cell death. Physiological Reviews 87, 99-163

Kruip TAM, denDaas JHG (1997): In vitro produced and cloned embryos: Effects on pregnancy, parturition and offspring. Theriogenology 47, 43-52

Kuhn DM, Arthur RE (1999): L-DOPA-quinone inactivates tryptophan hydroxylase and converts the enzyme to a redox-cycling quinoprotein. Molecular Brain Research 73, 78-84

Kumar S, Yedjou CG, Tchounwou PB (2014): Arsenic trioxide induces oxidative stress, DNA damage, and mitochondrial pathway of apoptosis in human leukemia (HL-60) cells. Journal of Experimental & Clinical Cancer Research 33, 42

Lambert C, Apel K, Biesalski HK, Frank J (2004): 2-Methoxyestradiol induces caspase-independent, mitochondria-centered apoptosis in DS-sarcoma cells. International Journal of Cancer 108, 493-501

Lane M, Gardner DK (1997): Differential regulation of mouse embryo development and viability by amino acids. Journal of Reproduction and Fertility 109, 153-164

Lane M, Maybach JM, Gardner DK (2002): Addition of ascorbate during cryopreservation stimulates subsequent embryo development. Human Reproduction 17, 2686-2693

Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD (2008): Dual roles of Nrf2 in cancer. Pharmacological Research 58, 262-270

Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C (2002): Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biology of Reproduction 67, 767-775

Lee HC, Wei YH (2005): Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. International Journal of Biochemistry & Cell Biology 37, 822-834

Lee KF, Chow JFC, Xu JS, Chan STH, Ip SM, Yeung WSB (2001): A comparative study of gene expression in murine embryos developed in vivo, cultured in vitro, and cocultured with human oviductal cells using messenger ribonucleic acid differential display. Biology of Reproduction 64, 910-917

Lee KS, Kim EY, Jeon K, Cho SG, Han YJ, Yang BC, Lee SS, Ko MS, Riu KJ, Lee HT, Park SP (2011): 3,4-Dihydroxyflavone acts as an antioxidant and antiapoptotic agent to support bovine embryo development in vitro. J Reprod Develop 57, 127-134

Lee MJ, Lee RK, Lin MH, Hwu YM (2012): Cleavage speed and implantation potential of early-cleavage embryos in IVF or ICSI cycles. Journal of Assisted Reproduction and Genetics 29, 745-750

Lee SK, Zhao MH, Kwon JW, Li YH, Lin ZL, Jin YX, Kim NH, Cui XS (2014): The association of mitochondrial potential and copy number with pig oocyte maturation and developmental potential. J Reprod Develop 60, 128-135

Leese HJ (2012): Metabolism of the preimplantation embryo: 40 years on. Reproduction 143, 417-427

Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG (2008): Metabolism of the viable mammalian embryo: quietness revisited. Molecular Human Reproduction 14, 667-672

Leibfried-Rutledge ML, Critser ES, Parrish JJ, First NL (1989): In vitro maturation and fertilization of bovine oocytes. Theriogenology 31 61–74

Leoni GG, Rosati I, Succu S, Bogliolo L, Bebbere D, Berlinguer F, Ledda S, Naitana S (2007): A low oxygen atmosphere during IVF accelerates the kinetic of formation of in vitro produced ovine blastocysts. Reproduction in Domestic Animals 42, 299-304

Lequarre AS, Feugang JM, Malhomme O, Donnay I, Massip A, Dessy F, Van Langendonckt A (2001): Expression of Cu/Zn and Mn superoxide dismutases during bovine embryo development: influence of in vitro culture. Molecular Reproduction and Development 58, 45-53

Leung L, Kwong M, Hou S, Lee C, Chan JY (2003): Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. The Journal of Biological Chemistry 278, 48021-48029

Li J, Foote RH, Simkin M (1993): Development of rabbit zygotes cultured in protein-free medium with catalase, taurine, or superoxide dismutase. Biology of Reproduction 49, 33-37

Li J, Ichikawa T, Jin Y, Hofseth LJ, Nagarkatti P, Nagarkatti M, Windust A, Cui T (2010): An essential role of Nrf2 in american ginseng-mediated anti-oxidative actions in cardiomyocytes. Journal of Ethnopharmacology 130, 222-230

Li RF, Wen LH, Wang SY, Bou S (2006): Development, freezability and amino acid consumption of bovine embryos cultured in synthetic oviductal fluid (SOF) medium containing amino acids at oviductal or uterine-fluid concentrations. Theriogenology 66, 404-414

Li XX, Lee KB, Lee JH, Kim KJ, Kim EY, Han KW, Park KS, Yu J, Kim MK (2014): Glutathione and cysteine enhance porcine preimplantation embryo development in vitro after intracytoplasmic sperm injection. Theriogenology 81, 309-314

Liebel F, Kaur S, Ruvolo E, Kollias N, Southall MD (2012): Irradiation of skin with visible light induces reactive oxygen species and matrix-degrading enzymes. Journal of Investigative Dermatology 132, 1901-1907

Liochev SI (1999): The mechanism of "Fenton-like" reactions and their importance for biological systems. A biologist's view. Metal Ions in Biological Systems, Vol 36 36, 1-39

Liu HC, He ZY, Mele C, Davis O, Rosenwaks Z (1997): Expression of apoptosis related genes in human oocytes, embryos, and reproductive cells. Fertility and Sterility O092-O092

Liu HC, He ZY, Mele CA, Veeck LL, Davis O, Rosenwaks Z (2000): Expression of apoptosis-related genes in human oocytes and embryos. Journal of Assisted Reproduction and Genetics 17, 521-533

Liu L, Keefe DL (2000): Cytoplasm mediates both development and oxidation-induced apoptotic cell death in mouse zygotes. Biology of Reproduction 62, 1828-1834

Liu Z, Foote RH (1995): Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O_2. Biology of Reproduction 53, 786-790

Livingston T, Eberhardt D, Lannett Edwards J, Godkin J (2004): Retinol improves bovine embryonic development in vitro. Reproductive Biology and Endocrinology 2, 83-89

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP (1999): Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. Journal of Reproduction and Fertility 117, 159-167

Lonergan P, Rizos D, Gutierrez-Adan A, Fair T, Boland MP (2003a): Oocyte and embryo quality: Effect of origin, culture conditions and gene expression patterns. Reproduction in Domestic Animals 38, 259-267

Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, de la Fuente J, Boland MP (2003b): Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. Biology of Reproduction 69, 1424-1431

Lonergan P, Woods A, Fair T, Carter F, Rizos D, Ward F, Quinn K, Evans A (2007): Effect of embryo source and recipient progesterone environment on embryo development in cattle. Reproduction Fertility and Development 19, 861-868

Lopes AS, Lane M, Thompson JG (2010): Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes. Human Reproduction 25, 2762-2773

Lopes S, Jurisicova A, Sun JG, Casper RF (1998): Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Human Reproduction 13, 896-900

Lotocki G, Keane RW (2002): Inhibitors of apoptosis proteins in injury and disease. Iubmb Life 54, 231-240

Loutradis D, John D, Kiessling AA (1987): Hypoxanthine causes a 2-cell block in random-bred mouse embryos. Biology of Reproduction 37, 311-316

Lu JM, Gong NA, Wang YC, Wang YX (2012): D-Amino acid oxidase-mediated increase in spinal hydrogen peroxide is mainly responsible for formalin-induced tonic pain. British Journal of Pharmacology 165, 1941-1955

Lu KH, Gordon I, Gallagher M, Mcgovern H (1987): Pregnancy established in cattle by transfer of embryos derived from invitro fertilization of oocytes matured invitro. Veterinary Record 121, 259-260

Luvoni GC, Keskintepe L, Brackett BG (1996): Improvement in bovine embryo production in vitro by glutathione-containing culture media. Molecular Reproduction and Development 43, 437-443

MacGarvey NC, Suliman HB, Bartz RR, Fu P, Withers CM, Welty-Wolf KE, Piantadosi CA (2012): Activation of mitochondrial biogenesis by heme oxygenase-1-mediated NF-E2-related factor-2 induction rescues mice from lethal Staphylococcus aureus sepsis. American Journal of Respiratory and Critical Care Medicine 185, 851-861

Machaty Z, Thompson JG, Abeydeera LR, Day BN, Prather RS (2001): Inhibitors of mitochondrial ATP production at the time of compaction improve development of in vitro produced porcine embryos. Molecular Reproduction and Development 58, 39-44

Malhotra JD, Kaufman RJ (2007): Endoplasmic reticulum stress and oxidative stress: A vicious cycle or a double-edged sword? Antioxidants & Redox Signaling 9, 2277-2293

Manes C, Lai NC (1995): Nonmitochondrial oxygen utilization by rabbit blastocysts and surface production of superoxide radicals. Journal of Reproduction and Fertility 104, 69-75

Manjunatha BM, Devaraj M, Gupta PSP, Ravindra JP, Nandi S (2009): Effect of taurine and melatonin in the culture medium on buffalo in vitro embryo development. Reproduction in Domestic Animals 44, 12-16

Many A, WesterhausenLarson A, KanbourShakir A, Roberts JM (1996): Xanthine oxidase/dehydrogenase is present in human placenta. Placenta 17, 361-365

Maroto R, PerezPolo JR (1997): BCL-2-related protein expression in apoptosis: Oxidative stress versus serum deprivation in PC12 cells. Journal of Neurochemistry 69, 514-523

Marti E, Marti JI, Muino-Blanco T, Cebrian-Perez JA (2008): Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. Journal of Andrology 29, 459-467

Matsuzawa N, Takamura T, Kurita S, Misu H, Ota T, Ando H, Yokoyama M, Honda M, Zen Y, Nakanuma Y, Miyamoto K, Kaneko S (2007): Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. Hepatology (St. Louis, MO) 46, 1392-1403

McCarthy MJ, Baumber J, Kass PH, Meyers SA (2010): Osmotic stress induces oxidative cell damage to Rhesus Macaque spermatozoa. Biology of Reproduction 82, 644-651

McEvoy TG, Robinson JJ, Sinclair KD (2001): Developmental consequences of embryo and cell manipulation in mice and farm animals. Reproduction 122, 507-518

McEvoy TG, Young LE, Wilmut I, Robinson JJ, Sinclair KD (2000): Large offspring syndrome and other consequences of ruminant embryo culture in vitro: relevance to blastocyst culture in human ART. Human Fertility (Cambridge). 3, 238-246

McKiernan SH, Bavister BD (2000): Culture of one-cell hamster embryos with water soluble vitamins: pantothenate stimulates blastocyst production. Human Reproduction 15, 157-164

Mello Filho AC, Hoffmann ME, Meneghini R (1984): Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. Biochemical Journal 218, 273-275

Melov S, Schneider JA, Day BJ, Hinerfeld D, Coskun P, Mirra SS, Crapo JD, Wallace DC (1998): A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. Nature Genetics 18, 159-163

Memili E, Dominko T, First NL (1998): Onset of transcription in bovine oocytes and preimplantation embryos. Molecular Reproduction and Development 51, 36-41

Merton J, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM, Dieleman SJ (2003): Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 59, 651-674

Merton JS, Vermeulen ZL, Otter T, Mullaart E, de Ruigh L, Hasler JF (2007): Carbon-activated gas filtration during in vitro culture increased pregnancy rate following transfer of in vitro-produced bovine embryos. Theriogenology 67, 1233-1238

Metcalfe AD, Hunter HR, Bloor DJ, Lieberman BA, Picton HM, Leese HJ, Kimber SJ, Brison DR (2004): Expression of 11 members of the BCL-2 family of apoptosis regulatory molecules during human preimplantation embryo development and fragmentation. Molecular Reproduction and Development 68, 35-50

Michael DD, Alvarez IM, Ocon OM, Powell AM, Talbot NC, Johnson SE, Ealy AD (2006): Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-tau production in bovine trophectoderm. Endocrinology 147, 3571-3579

Milei J, Ferreira R, Grana DR, Boveris A (2001): Oxidative stress and mitochondrial damage in coronary artery bypass graft surgery: effects of antioxidant treatments. Comprehensive Therapy 27, 108-116

Miller GF, Gliedt DW, Rakes JM, Rorie RW (1994): Addition of penicillamine, hypotaurine and epinephrine (Phe) or bovine oviductal epithelial-cells (Boec) alone or in combination to bovine in-vitro fertilization medium increases the subsequent embryo cleavage rate. Theriogenology 41, 689-696

Miquel J, Economos AC, Fleming J, Johnson JE (1980): Mitochondrial role in cell aging. Experimental Gerontology 15, 575-591

Misirlioglu M, Page GP, Sagirkaya H, Kaya A, Parrish JJ, First NL, Memili E (2006): Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. Proceedings of the National Academy of Sciences of the United States of America 103, 18905-18910

Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M (2009a): Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. Biology of Reproduction 80, 295-301

Mitchell M, Schulz SL, Armstrong DT, Lane M (2009b): Metabolic and mitochondrial dysfunction in early mouse embryos following maternal dietary protein intervention. Biology of Reproduction 80, 622-630

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011): ROS signaling: the new wave? Trends in Plant Science 16, 300-309

Moley KH, Chi MMY, Knudson CM, Korsmeyer SJ, Mueckler MM (1998): Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. Nature Medicine 4, 1421-1424

Monson R, Northey DL, Gottfredson R, Peschel DR, Rutledge JJ, Schaefer DM (1992): Pregnancy rates of in vitro produced bovine embryos following nonsurgical transfer. Theriogenology 37, 261-261

Moor ACE (2000): Signaling pathways in cell death and survival after photodynamic therapy. Journal of Photochemistry and Photobiology B-Biology 57, 1-13

Morales H, Tilquin P, Rees JF, Massip A, Dessy F, Van Langendonckt A (1999): Pyruvate prevents peroxide-induced injury of in vitro preimplantation bovine embryos. Molecular Reproduction and Development 52, 149-157

Moubasher AE, El Din AME, Ali ME, El-sherif WT, Gaber HD (2013): Catalase improves motility, vitality and DNA integrity of cryopreserved human spermatozoa. Andrologia 45, 135-139

Murphy MP (2009): How mitochondria produce reactive oxygen species. Biochemical Journal 417, 1-13

Muthukumar K, Kamath MS, Mangalaraj AM, Aleyamma T, Chandy A, George K (2013): Comparison of clinical outcomes following vitrified warmed day 5/6

blastocyst transfers using solid surface methodology with fresh blastocyst transfers. J Hum Reprod Sci 6, 59-64

Muzio M, Chinnaiyan AM, Kischkel FC, ORourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM (1996): FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85, 817-827

Mylonas C, Kouretas D (1999): Lipid peroxidation and tissue damage. In Vivo 13, 295-309

Nasresfahani M, Johnson MH, Aitken RJ (1990a): The effect of iron and iron chelators on the invitro block to development of the mouse preimplantation embryo - Bat6 a new medium for improved culture of mouse embryos invitro. Human Reproduction 5, 997-1003

Nasresfahani MH, Aitken JR, Johnson MH (1990b): Hydrogen-Peroxide levels in mouse oocytes and early cleavage stage embryos developed invitro or invivo. Development 109, 501-507

Nasresfahani MH, Johnson MH (1992): How does transferrin overcome the invitro block to development of the mouse preimplantation embryo. Journal of Reproduction and Fertility 96, 41-48

Nasresfahani MH, Winston NJ, Johnson MH (1992): Effects of glucose, glutamine, ethylenediaminetetraacetic acid and oxygen-tension on the concentration of reactive oxygen species and on development of the mouse preimplantation embryo invitro. Journal of Reproduction and Fertility 96, 219-231

Nasresfahani MM, Johnson MH (1991): The origin of reactive oxygen species in mouse embryos cultured invitro. Development 113, 551-560

Natsuyama S, Noda Y, Yamashita M, Nagahama Y, Mori T (1993): Superoxide dismutase and thioredoxin restore defective p34cdc2 kinase activation in mouse two-cell block. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1176, 90-94

Nerland DE (2007): The antioxidant/electrophile response element motif. Drug Metabolism Reviews 39, 235-248

Newby D, Marks L, Lyall F (2005): Dissolved oxygen concentration in culture medium: Assumptions and pitfalls. Placenta 26, 353-357

Nguyen KC, Willmore WG, Tayabali AF (2013): Cadmium telluride quantum dots cause oxidative stress leading to extrinsic and intrinsic apoptosis in hepatocellular carcinoma HepG2 cells. Toxicology 306, 114-123

Nguyen T, Huang HC, Pickett CB (2000): Transcriptional regulation of the antioxidant response element - activation by Nrf2 and repression by MafK. The Journal of Biological Chemistry 275, 15466-15473

Nguyen T, Nioi P, Pickett CB (2009): The Nrf2-Antioxidant response element signaling pathway and its activation by oxidative stress. The Journal of Biological Chemistry 284, 13291-13295

Nguyen T, Sherratt PJ, Pickett CB (2003): Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annual Review of Pharmacology and Toxicology 43, 233-260

Niemann H, Carnwath JW, Herrmann D, Wieczorek G, Lemme E, Lucas-Hahn A, Olek S (2010): DNA methylation patterns reflect epigenetic reprogramming in bovine embryos. Cellular Reprogramming 12, 33-42

Niemann H, Wrenzycki C (2000): Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: Implications for subsequent development. Theriogenology 53, 21-34

Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J, Mori T (1991): Involvement of superoxide radicals in the mouse 2-cell block. Molecular Reproduction and Development 28, 356-360

Nohl H, Hegner D (1978): Do mitochondria produce oxygen radicals invivo. European Journal of Biochemistry (Berlin) 82, 563-567

Nonogaki T, Noda Y, Narimoto K, Umaoka Y, Mori T (1992): Effects of superoxide dismutase on mouse in vitro fertilization and embryo culture system. Journal of Assisted Reproduction and Genetics 9, 274-280

Nonogaki T, Noda Y, Narimoto K, Umaoka Y, Mori T (1991): Protection from oxidative stress by thioredoxin and superoxide dismutase of mouse embryos fertilized in vitro. Human Reproduction 6, 1305-1310

Ocon-Grove OM, Cooke FNT, Alvarez IM, Johnson SE, Ott TL, Ealy AD (2008): Ovine endometrial expression of fibroblast growth factor (FGF) 2 and

conceptus expression of FGF receptors during early pregnancy. Domestic Animal Endocrinology 34, 135-145

Ohshima H, Bartsch H (1994): Chronic infections and inflammatory processes as cancer risk-factors - possible role of nitric-oxide in carcinogenesis. Mutation Research 305, 253-264

Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, Shibata T, Yamamoto M, Hirohashi S (2008): Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. Cancer Res 68, 1303-1309

Okada K, Shoda J, Taguchi K, Maher JM, Ishizaki K, Inoue Y, Ohtsuki M, Goto N, Sugimoto H, Utsunomiya H, Oda K, Warabi E, Ishii T, Yamamoto M (2009): Nrf2 counteracts cholestatic liver injury via stimulation of hepatic defense systems. Biochemical and Biophysical Research Communications 389, 431-436

Okado-Matsumoto A, Fridovich I (2001): Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. The Journal of Biological Chemistry 276, 38388-38393

Oller AR, Buser CW, Tyo MA, Thilly WG (1989): Growth of mammalian-cells at high oxygen concentrations. Journal of Cell Science 94, 43-49

Olson SE, Seidel GE (2000a): Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. Biology of Reproduction 62, 248-252

Olson SE, Seidel GE (2000b): Reduced oxygen tension and EDTA improve bovine zygote development in a chemically defined medium. Journal of Animal Science 78, 152-157

Orsi NM, Leese HJ (2001): Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase, superoxide dismutase and pyruvate. Molecular Reproduction and Development 59, 44-53

Osburn WO, Kensler TW (2008): Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. Mutation Research 659, 31-39

Oyawoye O, Gadir AA, Garner A, Constantinovici N, Perrett C, Hardiman P (2003): Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. Human Reproduction 18, 2270-2274

Ozawa M, Nagai T, Fahrudin M, Karja NW, Kaneko H, Noguchi J, Ohnuma K, Kikuchi K (2006): Addition of glutathione or thioredoxin to culture medium reduces intracellular redox status of porcine IVM/IVF embryos, resulting in improved development to the blastocyst stage. Molecular Reproduction and Development 73, 998-1007

Ozgur R, Turkan I, Uzilday B, Sekmen AH (2014): Endoplasmic reticulum stress triggers ROS signalling, changes the redox state, and regulates the antioxidant defence of Arabidopsis thaliana. Journal of Experimental Botany 65, 1377-1390

Pampfer S, Wuu YD, Vanderheyden I, Dehertogh R (1994): Expression of tumor-necrosis-factor-alpha (Tnf-Alpha) receptors and selective effect of Tnf-Alpha on the inner cell mass in mouse blastocysts. Endocrinology 134, 206-212

Papis K, Poleszczuk O, Wenta-Muchalska E, Modlinski JA (2007): Melatonin effect on bovine embryo development in vitro in relation to oxygen concentration. Journal of Pineal Research 43, 321-326

Parchment RE, Lewellyn A, Swartzendruber D, Pierce GB (1990): Serum amine oxidase activity contributes to crisis in mouse embryo cell-lines. Proceedings of the National Academy of Sciences of the United States of America 87, 4340-4344

Parrish JJ, Susko-Parrish J, Winer MA, First NL (1988): Capacitation of bovine sperm by heparin. Biology of Reproduction 38, 1171-1180

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

Pascoe GA, Fariss MW, Olafsdottir K, Reed DJ (1987): A role of vitamin E in protection against cell injury. Maintenance of intracellular glutathione precursors and biosynthesis. European Journal of Biochemistry (Berlin) 166, 241-247

Pasqualotto EB, Agarwal A, Sharma RK, Izzo VM, Pinotti JA, Joshi NJ, Rose BI (2004): Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertility and Sterility 81, 973-976

Paszkowski T, Clarke RN (1996): Antioxidative capacity of preimplantation embryo culture medium declines following the incubation of poor quality embryos. Human Reproduction 11, 2493-2495

Piantadosi CA, Carraway MS, Babiker A, Suliman HB (2008): Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. Circulation Research 103, 1232-1240

Pierce GB, Parchment RE, Lewellyn AL (1991): Hydrogen-Peroxide as a mediator of programmed cell-death in the blastocyst. Differentiation 46, 181-186

Pitt RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A (1996): Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. The Journal of Biological Chemistry 271, 12687-12690

Plante L, Plante C, Shepherd DL, King WA (1994): Cleavage and 3H-uridine incorporation in bovine embryos of high in vitro developmental potential. Molecular Reproduction and Development 39, 375-383

Powers SK, Jackson MJ (2008): Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiological Reviews 88, 1243-1276

Prasad CS, Palanisamy A, Gomathy VS, Satheshkumar S, Thangavel A, Raj GD (2013): Effect of Tcm-199 and synthetic oviductal fluid (SOF) medium and cysteamine supplementation to in vitro maturation media on maturation, cleavage rate and subsequent embryonic. Buffalo Bulletin 32, 182-188

Qiu XL, Brown K, Hirschey MD, Verdin E, Chen D (2010): Calorie restriction reduces oxidative stress by SIRT3-Mediated SOD2 activation. Cell Metabolism 12, 662-667

Quinn PJ (2004): Is the distribution of alpha-tocopherol in membranes consistent with its putative functions? Biochem (Mosc) Suppl Ser A Membr Cell Biol 69, 58-66

Radermacher KA, Wingler K, Langhauser F, Altenhofer S, Kleikers P, Hermans JJR, de Angelis MH, Kleinschnitz C, Schmidt HHHW (2013): Neuroprotection after stroke by targeting NOX4 as a source of oxidative stress. Antioxidants & Redox Signaling 18, 1418-1427

Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, Dhama K (2014): Oxidative stress, prooxidants, and antioxidants: The interplay. Biomed Research International

Rahimi G, Isachenko E, Sauer H, Isachenko V, Wartenberg M, Hescheler J, Mallmann P, Nawroth F (2003): Effect of different vitrification protocols for human ovarian tissue on reactive oxygen species and apoptosis. Reprod Fertil Dev 15, 343-349

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

Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W (1992): Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry (Berlin) 97, 493-497

Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW (2001): Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 98, 3410-3415

Rao MS, Reddy JK (2001): Peroxisomal beta-oxidation and steatohepatitis. Seminars in Liver Disease 21, 43-55

Rashid K, Das J, Sil PC (2013): Taurine ameliorate alloxan induced oxidative stress and intrinsic apoptotic pathway in the hepatic tissue of diabetic rats. Food and Chemical Toxicology 51, 317-329

Ravanat JL, Di Mascio P, Martinez GR, Medeiros MHG, Cadet J (2000): Singlet oxygen induces oxidation of cellular DNA. The Journal of Biological Chemistry 275, 40601-40604

Rego AC, Oliveira CR (2003): Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: Implications for the pathogenesis of neurodegenerative diseases. Neurochemical Research 28, 1563-1574

Richter C, Park JW, Ames BN (1988): Normal oxidative damage to mitochondrial and nuclear-DNA is extensive. Proceedings of the National Academy of Sciences of the United States of America 85, 6465-6467

Rieger D, McGowan LT, Cox SF, Pugh PA, Thompson JG (2002): Effect of 2,4-dinitrophenol on the energy metabolism of cattle embryos produced by in vitro fertilization and culture. Reproduction Fertility and Development 14, 339-343

Riley JK, Heeley JM, Wyman AH, Schlichting EL, Moley KH (2004): TRAIL and KILLER are expressed and induce apoptosis in the murine preimplantation embryo. Biology of Reproduction 71, 871-877

Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM (2006): Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertility and Sterility 86, 1252-1265, 1265 e1251-1236

Ritchie A, Gutierrez O, Fernandez-Luna JL (2009): PAR bZIP-bik is a novel transcriptional pathway that mediates oxidative stress-induced apoptosis in fibroblasts. Cell Death and Differentiation 16, 838-846

Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P (2002a): Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. Molecular Reproduction and Development 62, 320-327

Rizos D, Gutierrez-Adan A, Perez-Garnelo S, de la Fuente J, Boland MP, Lonergan P (2003): Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression. Biology of Reproduction 68, 236-243

Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, de la Fuente J, Gutierrez-Adan A (2002b): Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. Biology of Reproduction 66, 589-595

Rizos D, Ward F, Boland MP, Lonergan P (2001): Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. Theriogenology 56, 1-16

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P (2002c): Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in

vivo: Implications for blastocyst yield and blastocyst quality. Molecular Reproduction and Development 61, 234-248

Rock CL, Jacob RA, Bowen PE (1996): Update on the biological characteristics of the antioxidant micronutrients: Vitamin C, vitamin E, and the carotenoids. Journal of the American Dietetic Association 96, 693-702

Rooke JA, McEvoy TG, Ashworth CJ, Robinson JJ, Wilmut I, Young LE, Sinclair KD (2007): Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. Theriogenology 67, 639-647

Rosenkrans CF, Jr., First NL (1994): Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. Journal of Animal Science 72, 434-437

Rosenthal MD, Glew H (2009): Medical biochemistry: Human metabolism in health and disease Biochemical Education 440

Roushandeh AM, Pasbakhsh P, Afizadeh Z, Roudkenai MH (2007): In vitro maturation media, cysteamine concentration and glutathione level affect blstocysts development in mouse. Iranian Journal of Reproductive Medicine 5, 159-163

Routledge MN, Wink DA, Keefer LK, Dipple A (1994): DNA-Sequence changes induced by 2 nitric-oxide donor drugs in the supf assay. Chemical Research in Toxicology 7, 628-632

Rozell MD, Williams JE, Butler JE (1992): Changes in concentration of adenosine-triphosphate and adenosine-diphosphate in individual preimplantation sheep embryos. Biology of Reproduction 47, 866-870

Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P (2004): Toxic proteins released from mitochondria in cell death. Oncogene (London) 23, 2861-2874

Sahin E, DePinho RA (2010): Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 464, 520-528

Saini M, Selokar NL, Agrawal H, Singla SK, Chauhan MS, Manik RS, Palta P (2014): Low oxygen tension improves developmental competence and reduces apoptosis in hand-made cloned buffalo (Bubalus bubalis) embryos. Livest Sci In press,

Saito A, Hayashi T, Okuno S, Nishi T, Chan PH (2004): Oxidative stress is associated with XIAP and Smac/DIABLO signaling pathways in mouse brains after transient focal cerebral ischemia. Stroke 35, 1443-1448

Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR, Thomas AJ (2003): Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertility and Sterility 79, 1597-1605

Sangild PT, Schmidt M, Jacobsen H, Fowden AL, Forhead A, Avery B, Greve T (2000): Blood chemistry, nutrient metabolism, and organ weights in fetal and newborn calves derived from in vitro-produced bovine embryos. Biology of Reproduction 62, 1495-1504

Santos AN, Korber S, Kullertz G, Fischer G, Fischer B (2000): Oxygen stress increases prolyl cis/trans isomerase activity and expression of cyclophilin 18 in rabbit blastocysts. Biology of Reproduction 62, 1-7

Santos CXC, Tanaka LY, Wosniak J, Laurindo FRM (2009): Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: Roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. Antioxidants & Redox Signaling 11, 2409-2427

Sarkar A, Das J, Manna P, Sil PC (2011): Nano-copper induces oxidative stress and apoptosis in kidney via both extrinsic and intrinsic pathways. Toxicology 290, 208-217

Sastre J, Pallardo FV, Vina J (2000): Mitochondrial oxidative stress plays a key role in aging and apoptosis. Iubmb Life 49, 427-435

Sata R, Tsujii H, Yamashita S, Hoshi H (1999): Fatty acid composition of bovine embryos cultured in serum-free and serum-containing medium during early embryonic development. Journal of Reproduction and Development 45, 97–103

Sato E, Mokudai T, Niwano Y, Kohno M (2011): Kinetic analysis of reactive oxygen species generated by the in vitro reconstituted NADPH oxidase and xanthine oxidase systems. Journal of Biochemistry 150, 173-181

Scalbert A, Johnson IT, Saltmarsh M (2005): Polyphenols: antioxidants and beyond. American Journal of Clinical Nutrition 81, 215s-217s

Schonfeld P, Wieckowski MR, Lebiedzinska M, Wojtczak L (2010): Mitochondrial fatty acid oxidation and oxidative stress: Lack of reverse electron transfer-associated production of reactive oxygen species. Biochimica Et Biophysica Acta-Bioenergetics 1797, 929-938

Schrader M, Fahimi HD (2004): Mammalian peroxisomes and reactive oxygen species. Histochemistry and Cell Biology 122, 383-393

Schrader M, Fahimi HD (2006): Peroxisomes and oxidative stress. Biochimica Et Biophysica Acta-Molecular Cell Research 1763, 1755-1766

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME (1998): Apoptosis signaling by death receptors. European Journal of Biochemistry 254, 439-459

Schweigert FJ, Zucker H (1988): Concentrations of vitamin A, beta-carotene and vitamin E in individual bovine follicles of different quality. Journal of Reproduction and Fertility 82, 575-579

Sekiya M, Hiraishi A, Touyama M, Sakamoto K (2008): Oxidative stress induced lipid accumulation via SREBP1c activation in HepG2 cells. Biochemical and Biophysical Research Communications 375, 602-607

Shannon P (1978): Factors affecting semen preservation and conception rates in cattle. Journal of Reproduction and Fertility 54, 519-527

Shapiro BS, Richter KS, Harris DC, Daneshmand ST (2001): A comparison of day 5 and day 6 blastocyst transfers. Fertility and Sterility 75, 1126-1130

Sharma RK, Said T, Agarwal A (2004): Sperm DNA damage and its clinical relevance in assessing reproductive outcome. Asian Journal of Andrology 6, 139-148

Shekarriz M, Dewire DM, Thomas AJ, Agarwal A (1995a): A method of human semen centrifugation to minimize the Iatrogenic sperm injuries caused by reactive oxygen species. European Urology 28, 31-35

Shekarriz M, Sharma RK, Thomas AJ, Agarwal A (1995b): Positive myeloperoxidase staining (Endtz Test) as an indicator of excessive reactive oxygen species formation in semen. Journal of Assisted Reproduction and Genetics 12, 70-74

Shen HR, Spikes JD, Smith CJ, Kopecek J (2000): Photodynamic cross-linking of proteins - V. Nature of the tyrosine-tyrosine bonds formed in the FMN-sensitized intermolecular cross-linking of N-acetyl-L-tyrosine. Journal of Photochemistry and Photobiology a-Chemistry 133, 115-122

Shigenaga MK, Hagen TM, Ames BN (1994): Oxidative damage and mitochondrial decay in aging. Proceedings of the National Academy of Sciences of the United States of America 91, 10771-10778

Shimizu Y, Miyakura R, Otsuka Y (2014): Nuclear receptor subfamily 4, group A, member 1 inhibits extrinsic apoptosis and reduces caspase-8 activity in H2O2-induced human HUC-F2 fibroblasts. Redox Rep [Epub ahead of print],

Shokolenko I, Venediktova N, Bochkareva A, Wilson GL, Alexeyev MF (2009): Oxidative stress induces degradation of mitochondrial DNA. Nucleic Acids Research 37, 2539-2548

Sies H (1986): Biochemistry of oxidative stress. Angewandte Chemie-International Edition in English 25, 1058-1071

Singh I (1996): Mammalian peroxisomes: Metabolism of oxygen and reactive oxygen species. Annals of the New York Academy of Sciences (New York, NY) 804, 612-627

Somfai T, Kaneda M, Akagi S, Watanabe S, Haraguchi S, Mizutani E, Dang-Nguyen TQ, Geshi M, Kikuchi K, Nagai T (2011): Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. Reprod Fertil Dev 23, 912-920

Soto P, Natzke RP, Hansen PJ (2003): Actions of tumor necrosis factor-alpha on oocyte maturation and embryonic development in cattle. American Journal of Reproductive Immunology 50, 380-388

Spanos S, Rice S, Karagiannis P, Taylor D, Becker DL, Winston RML, Hardy K (2002): Caspase activity and expression of cell death genes during development of human preimplantation embryos. Reproduction 124, 353-363

Spector AA, Santos EC, Fletcher JE, Ashbrook JD (1972): Quantitative analysis of uptake of free fatty-acid by mammalian-cells - lauric acid and human erythrocytes. Journal of Lipid Research 13, 445-&

Spector AA, Soboroff JM (1972): Studies on cellular mechanism of free fatty-acid uptake using an analog, hexadecanol. Journal of Lipid Research 13, 790-&

Sudano MJ, Mattos MCC, Fernandes CB, Mazieiro RR, Landim-Alvarenga FC (2010): In vitro production of bovine embryos using Sigma antioxidant supplement®, α-tocopherol and L-ascorbic acid. Animal Reproduction 7, 42-48

Sudano MJ, Paschoal DM, Rascado Tda S, Magalhaes LC, Crocomo LF, de Lima-Neto JF, Landim-Alvarenga Fda C (2011): Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. Theriogenology 75, 1211-1220

Sudano MJ, Santos VG, Tata A, Ferreira CR, Paschoal DM, Machado R, Buratini J, Eberlin MN, Landim-Alvarenga FDC (2012): Phosphatidylcholine and sphingomyelin profiles vary in bos taurus indicus and bos taurus taurus in vitro- and in vivo-produced blastocysts. Biology of Reproduction 87,

Sugino N, Takiguchi S, Kashida S, Karube A, Nakamura Y, Kato H (2000): Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. Molecular Human Reproduction 6, 19-25

Suzuki T, Sumantri C, Khan NHA, Murakami M, Saha S (1999): Development of a simple, portable carbon dioxide incubator for in vitro production of bovine embryos. Animal Reproduction Science 54, 149-157

Taanman JW (1999): The mitochondrial genome: structure, transcription, translation and replication. Biochimica Et Biophysica Acta-Bioenergetics 1410, 103-123

Tahaei LS, Eimani H, Yazdi PE, Ebrahimi B, Fathi R (2011): Effects of retinoic acid on maturation of immature mouse oocytes in the presence and absence of a granulosa cell co-culture system. Journal of Assisted Reproduction and Genetics 28, 553-558

Takahashi M (2012): Oxidative stress and redox regulation on in vitro development of mammalian embryos. Journal of Reproduction and Development 58, 1-9

Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N, Okano A (1993): Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos. Biology of Reproduction 49, 228-232

Takahashi M, Nagai T, Okamura N, Takahashi H, Okano A (2002): Promoting effect of beta-mercaptoethanol on in vitro development under oxidative stress and cystine uptake of bovine embryos. Biology of Reproduction 66, 562-567

Tamura H, Takasaki A, Miwa I, Tanoguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ, Sugino N (2008): Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. Journal of Pineal Research 44, 280-287

Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y, Sugino N (2012): The role of melatonin as an antioxidant in the follicle. Journal of Ovarian Research 5,

Tanaka Y, Aleksunes LM, Yeager RL, Gyamfi MA, Esterly N, Guo GL, Klaassen CD (2008): NF-E2-related factor 2 inhibits lipid accumulation and oxidative stress in mice fed a high-fat diet. Journal of Pharmacology and Experimental Therapeutics 325, 655-664

Tao RD, Coleman MC, Pennington JD, Ozden O, Park SH, Jiang HY, Kim HS, Flynn CR, Hill S, McDonald WH, Olivier AK, Spitz DR, Gius D (2010): Sirt3-Mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. Molecular Cell 40, 893-904

Tarazona AM, Rodriguez JI, Restrepo LF, Olivera-Angel M (2006): Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. Reproduction in Domestic Animals 41, 5-11

Tarin JJ, Trounson AO (1993): Effects of stimulation or inhibition of lipid-peroxidation on freezing-thawing of mouse embryos. Biology of Reproduction 49, 1362-1368

Tarin JJ, Vendrell FJ, Ten J, Blanes R, van Blerkom J, Cano A (1996): The oxidizing agent tertiary butyl hydroperoxide induces disturbances in spindle organization, c-meiosis, and aneuploidy in mouse oocytes. Molecular Human Reproduction 2, 895-901

Taylor K, Roberts P, Sanders K, Burton P (2009): Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. Reproductive Biomedicine Online 18, 184-189

Thomas M, Jain S, Kumar GP, Laloraya M (1997): A programmed oxyradical burst causes hatching of mouse blastocysts. Journal of Cell Science 110, 1597-1602

Thompson JG, Allen NW, McGowan LT, Bell ACS, Lambert MG, Tervit HR (1998): Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. Theriogenology 49, 1239-1249

Thompson JG, McNaughton C, Gasparrini B, McGowan LT, Tervit HR (2000): Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction

and blastulation of bovine embryos cultured in vitro. Journal of Reproduction and Fertility 118, 47-55

Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ (1996): Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. Journal of Reproduction and Fertility 106, 299-306

Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR (1990): Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. Journal of Reproduction and Fertility 89, 573-578

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM (2009): Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Human Reproduction 24, 2061-2070

Tomek W, Sterza FAM, Kubelka M, Wollenhaupt K, Torner H, Anger M, Kanitz W (2002): Regulation of translation during in vitro maturation of bovine oocytes: The role of MAP kinase, eIF4E (cap binding protein) phosphorylation, and eIF4E-BP1. Biology of Reproduction 66, 1274-1282

Trimarchi JR, Liu L, Porterfield DM, Smith PJS, Keefe DL (2000): Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. Biology of Reproduction 62, 1866-1874

Tripathi A, Shrivastav TG, Chaube SK (2012): Aqueous extract of Azadirachta indica (neem) leaf induces generation of reactive oxygen species and mitochondria-mediated apoptosis in rat oocytes. Journal of Assisted Reproduction and Genetics 29, 15-23

Tu BP, Weissman JS (2004): Oxidative protein folding in eukaryotes: mechanisms and consequences. Journal of Cell Biology 164, 341-346

Turrens JF (2003): Mitochondrial formation of reactive oxygen species. Journal of Physiology-London 552, 335-344

Twigg JP, Irvine DS, Aitken RJ (1998): Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Human Reproduction 13, 1864-1871

Uhm SJ, Gupta MK, Yang JH, Lee SH, Lee HT (2007): Selenium improves the reduces the apoptosis developmental ability and in porcine parthenotes. Molecular Reproduction and Development 74, 1386-1394

Ungvari Z, Sonntag WE, de Cabo R, Baur JA, Csiszar A (2011): Mitochondrial protection by resveratrol. Exercise and Sport Sciences Reviews 39, 128-132

Van Blerkom J, Cox H, Davis P (2006): Regulatory roles for mitochondria in the peri-implantation mouse blastocyst: possible origins and developmental significance of differential Delta Psi m. Reproduction 131, 961-976

Van Buul JD, Fernandez-Borja M, Anthony EC, Hordijk PL (2005): Expression and localization of NOX2 and NOX4 in primary human endothelial cells. Antioxidants & Redox Signaling 7, 308-317

Van Guilder GP, Hoetzer GL, Greiner JJ, Stauffer BL, Desouza CA (2006): Influence of metabolic syndrome on biomarkers of oxidative stress and inflammation in obese adults. Obesity (Silver Spring) 14, 2127-2131

Van Soom A, Van Vlaenderen I, Mahmoudzadeh AR, Deluyker H, de Kruif A (1992): Compaction rate of in vitro fertilized bovine embryos related to the interval from insemination to first cleavage. Theriogenology 38, 905-919

Vanblerkom J, Davis PW (1994): Cytogenetic, cellular, and developmental consequences of cryopreservation of immature and mature mouse and human oocytes. Microscopy Research and Technique 27, 165-193

Varisanga MD, Dong YJ, Mtango NR, Suzuki T (2002): Comparison of the effects of using standard and simple portable CO_2 incubators on the in vitro developmental competence of bovine embryos reconstituted by somatic cell nuclear transfer. Theriogenology 58, 77-86

Vaux DL, Silke J (2003): Mammalian mitochondrial IAP binding proteins. Biochem Bioph Res Co 304, 499-504

Verhagen AM, Kratina TK, Hawkins CJ, Silke J, Ekert PG, Vaux DL (2007): Identification of mammalian mitochondrial proteins that interact with IAPs via N-terminal IAP binding motifs. Cell Death and Differentiation 14, 348-357

Vigneault C, Gravel C, Vallee M, McGraw S, Sirard MA (2009): Unveiling the bovine embryo transcriptome during the maternal-to-embryonic transition. Reproduction 137, 245-257

Villeneuve NF, Sun Z, Chen W, Zhang DD (2009): Nrf2 and p21 regulate the fine balance between life and death by controlling ROS levels. Cell Cycle 8, 3255-3256

Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H, Thomsen PD (1999): A high proportion of bovine blastocysts produced in vitro are mixoploid. Biology of Reproduction 60, 1273-1278

Vonzglinicki T, Saretzki G, Docke W, Lotze C (1995): Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts - a model for senescence. Experimental Cell Research 220, 186-193

Wakefield SL, Lane M, Mitchell M (2011): Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. Biology of Reproduction 84, 572-580

Wakschlag LS, Hans SL, Bertolini M, Bertolini LR, Gerger RPC, Batchelder CA, Anderson GB (2007): Developmental problems during pregnancy after in vitro embryo manipulations. Revista Brasileira De Reprodução Animal 31, 391-405

Wang CX, Youle RJ (2009): The role of mitochondria in apoptosis. Annual Review of Genetics 43, 95-118

Wang LY, Wang DH, Zou XY, Xu CM (2009): Mitochondrial functions on oocytes and preimplantation embryos. Journal of Zhejiang University-Science B 10, 483-492

Wang X, Falcone T, Attaran MD, Goldberg JM, Agarwal A, Sharma RK (2002): Vitamin C and Vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. Fertility and Sterility 78, 1272-1277

Wang ZG, Yu SD, Xu ZR (2007): Effect of supplementation of green tea polyphenols on the developmental competence of bovine oocytes in vitro. Brazilian Journal of Medical and Biological Research 40, 1079-1085

Wells PG, McCallum GP, Chen CS, Henderson JT, Lee CJJ, Perstin J, Preston TJ, Wiley MJ, Wong AW (2009): Oxidative stress in developmental origins of disease: Teratogenesis, neurodevelopmental deficits, and cancer. Toxicological Sciences 108, 4-18

Wild AC, Moinova HR, Mulcahy RT (1999): Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. The Journal of Biological Chemistry 274, 33627-33636

Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G (2001): Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. Human Reproduction 16, 909-917

Winterbourn CC (1995): Free-Radical toxicology and antioxidant defense. Clinical and Experimental Pharmacology and Physiology 22, 877-880

Wiseman H, Halliwell B (1996): Damage to DNA by reactive oxygen and nitrogen species: Role in inflammatory disease and progression to cancer. Biochemical Journal 313, 17-29

Wrenzycki C, Herrmann D, Keskintepe L, Martins A, Jr., Sirisathien S, Brackett B, Niemann H (2001): Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. Human Reproduction 16, 893-901

Wu D, Cederbaum AI (2003): Alcohol, oxidative stress, and free radical damage. Alcohol Research & Health 27, 277-284

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS (1998): Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Human Reproduction 13, 998-1002

Yeh CT, Yen GC (2006): Induction of hepatic antioxidant enzymes by phenolic acids in rats is accompanied by increased levels of multidrug resistance-associated protein 3 mRNA expression. Journal of Nutrition 136, 11-15

You J, Kim J, Lim J, Lee E (2010): Anthocyanin stimulates in vitro development of cloned pig embryos by increasing the intracellular glutathione level and inhibiting reactive oxygen species. Theriogenology 74, 777-785

Young LE, Sinclair KD, Wilmut I (1998): Large offspring syndrome in cattle and sheep. Reviews of Reproduction 3, 155-163

Yuan YQ, Van Soom A, Coopman FO, Mintiens K, Boerjan ML, Van Zeveren A, de Kruif A, Peelman LJ (2003): Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. Theriogenology 59, 1585-1596.

Yuh HS, Yu DH, Shin MJ, Kim HJ, Bae KB, Lee DS, Lee HC, Chang WK, Park SB, Lee SG, Park HD, Ha JH, Hyun BH, Ryoo ZY (2010): The effects of various

antioxidants on the development of parthenogenetic porcine embryos. In Vitro Cellular & Developmental Biology-Animal 46, 148-154

Yuzefovych LV, Musiyenko SI, Wilson GL, Rachek LI (2013): Mitochondrial DNA damage and dysfunction, and oxidative stress are associated with endoplasmic reticulum stress, protein degradation and apoptosis in high fat diet-induced insulin resistance mice. Plos One 8,

Zeevalk GD, Bernard LP, Song C, Gluck M, Ehrhart J (2005): Mitochondrial inhibition and oxidative stress: Reciprocating players in neurodegeneration. Antioxidants & Redox Signaling 7, 1117-1139

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

Zhao X, Du W, Wang D, Hao H, Qin T, Liu Y, Zhu H (2012): Controlled freezing and open-pulled straw (OPS) vitrification of in vitro produced bovine blastocysts following analysis of ATP content and reactive oxygen species (ROS) level. Journal of Integrative Agriculture 11, 446–455

Zheng SX, Newton GL, Gonick G, Fahey RC, Ward JF (1988): Radioprotection of DNA by thiols - relationship between the net charge on a thiol and its ability to protect DNA. Radiation Research 114, 11-27

Zhu H, Itoh K, Yamamoto M, Zweier JL, Li YB (2005): Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: Protection against reactive oxygen and nitrogen species-induced cell injury. Febs Lett 579, 3029-3036

Zoeteweij JP, Vandewater B, Debont HJGM, Mulder GJ, Nagelkerke JF (1992): Involvement of intracellular Ca^{2+} and K^{+} in dissipation of the mitochondrial-membrane potential and cell-death induced by extracellular ATP in hepatocytes. Biochemical Journal 288, 207-213

Zorn B, Vidmar G, Meden-Vrtovec H (2003): Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection. International Journal of Andrology 26, 279-285

Zribi N, Chakroun NF, Ben Abdallah F, Elleuch H, Sellami A, Gargouri J, Rebai T, Fakhfakh F, Keskes LA (2012): Effect of freezing-thawing process and

quercetin on human sperm survival and DNA integrity. Cryobiology 65, 326-331

Zuo SJ, Boorstein RJ, Teebor GW (1995): Oxidative damage to 5-methylcytosine in DNA. Nucleic Acids Research 23, 3239-3243

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4. Publications

Amin A, Gad A, Salilew-Wondim D, Prastowo S, Held E, Hoelker M, Rings F, Tholen E, Uddin J, Looft C, Schellander K, Tesfaye D. Bovine embryo survival under ~~oxidative~~ stress condition is associated with the activity of NRF2 mediated oxidative stress response p'athway. Molecular Reproduction and Development, 81: 497–513, 2014.

Amin A, Held E, Gad A, Salilew-Wondim D, Prastowo S, Hoelker M, Rings F,Tholen E, Udin J, Looft C, Schellander K, Tesfaye D. Activity of NRF2 Mediated Oxidative ~~Stress R~~esponse and Embryo Survival Under Oxidative Stress Condition (Abstract). 4th mammalian embryo genomics meeting. Quebec, Canada.09-11.10.2013**. pp.66**

Prastowo S, **Amin A,** Rings F, Salilew-Wondim D, Uddin MJ, Tholen E, Looft C, Schellander K, Tesfaye D, Hölker M. Expression of AMPK and Lipid Metabolism Related Gen~~es In Bo~~vine Embryo Cultured With and Without Serum **(Abstract)** .DGfZ-Jahrestagung und DGfZ-/GfT Gemeinschaftstagung, Georg-August-Universität Göttingen, Germany. 4-5.09.2013

Dawit Tesfaye, **Ahmed Amin**, Eva Held, Ahmed Gad, Dessie Salilew-Wondim, Sigit Prastowo, Micheal Hoelker, Franca Rings, Ernst Tholen, Jasim Udin, Christian Looft, Karl Schellande~~r . Activity of~~ NRF2-Mediated Oxidative Stress Response in Bovine Embryos **(Abstract).** Society for the Study of Reproduction (SSR's) 46th annual meeting, Montréal, Québec, Canada. 22–26.07.2013

Amin A, Held E, Gad A, Salilew-Wondim D, Prastowo S, Hoelker M, Rings F,Tholen E, Udin J, Looft C, Schellander K, Tesfaye D.Expression analysis of Nrf2 mediated ~~oxidative~~ stress response and lipid metabolism genes in early stages of bovine embryo development**(Abstract)**. DGfZ-Jahrestagung, Institut für Agrar- und Ernährungswissenschaften der Martin-Luther-Universität, Halle / Saale, Germany. 12-13.09.2012

Abdel-Salam, S. A., W. Mekkawy, Y. M. Hafez, **A. A. Zaki**, and S. Abou-Bakr, "Fitting lactation curve of Egyptian buffalo using three different models.", Proceedings of the 3rd International Conference on sustainable animal agriculture for developing countries, Nakhon Ratchasima, Thailand, Suranaree University of Technology, pp. 737-741, 2011.

M. S. Hassanane**, A. A. Zaki**, S. Abou-Bakr, RA. A. Nigm. Genetic polymorphism of some microsatellites on chromosome seven in the egyptian buffalo. Egyptian Journal of Animal Production. Giza, Egypt. 44(2):97-110.2007