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Enhancing the developmental competence of bovine oocytes and embryos

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Declaration

I hereby declare that all the studies in this thesis is my own work, except were acknowledgement is made by reference. The work described here has not been submitted anywhere for any other degree of qualification. All assistance given to me during the preparation of this thesis is also acknowledged.

Haitham O. Alhilfi

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Abstract

Enhancing the developmental competence of oocytes and quality of embryos is central to the success of *in vitro* embryo production (IVP) both in human and animal laboratories. However, there is currently no optimal protocol for IVP in either a commercial or clinical setting that leads to high yields of developmentally competent embryos and pregnancy outcomes. The current series of studies were performed to establish the effects of nitric oxide donor (Sodium nitroprusside (SNP)) and the proteasome inhibitor (MG132) on nuclear maturation and mitochondrial activity of bovine oocytes. Also, to establish the effects of Transforming Growth Factor B1 (TGFB1) and Colony Stimulating Factor 2 (CSF2) added to embryo culture (IVC) on the development of bovine embryos beyond morulation.

In the first series of experiments, 10 μM sodium nitroprusside delayed polar body (PB) extrusion (P=0.013). Also, 10 μM MG132 reduced PB extrusion (P=0.003), ATP content in oocytes (P=0.003) and the proportion of zygotes reaching the blastocyst stage (P=0.001). However, neither SNP nor MG132 had any effect on mtDNA copy number. MG132 but not SNP reduced (P=0.001) the proportion of cleaved zygotes reaching blastocyst at D8 of culture.

In the second series of experiments, utilizing different concentrations of MG132 revealed that 10 μM MG132 reduced (P=0.003) the proportion of ≥4-cell embryos at D2 of IVC. On the other hand, truncated (8 h) incubation of cumulus-oocyte complexes (COCs) with sperm reduced (P=0.003) the number of cleaved zygotes but increased (P<0.001) ≥4-cell embryos at D2 of IVC. Treatment with MG132 late in maturation followed by 8 h incubation period of gametes resulted in increase (P=0.013) of cleaved zygotes at D2 of IVC and increases (P=0.007) blastocysts of inseminated oocytes, and (P=0.004) of cleaved zygotes, in comparison with 18 h IVF counterpart.

In the third series of experiments, a combination of TGFB1 and CSF2 reduced (P=0.016) the proportion of cleaved zygotes reaching the blastocyst stage but had no effect on total cell number in the resultant blastocysts. Also, TGFB1 from D5 eliminated (P=0.025) the positive effect of CSF2 on embryonic development. Furthermore, only CSF2 added separately increased (P=0.004) the proportion of blastocysts and cell within the ICM for early and late blastocysts. However, treatment of embryos with either 10 or 50 ng ml^{-1} TGFB1 from D2 of IVC had no effect on *SMAD* signalling in resultant D7 embryos.

In the fourth and final series of experiments, embryonic development were enhanced when MG132 was included during late maturation followed by 8 h IVF and CSF2 from D5 of IVC (P=0.039). On the other hand, applying the latter treatment regime skewed (P=0.05) blastocyst sex ratio in favour of females.

Accordingly, utilizing MG132 during late maturation has beneficial effects but only when followed by a short period of incubation of gametes during fertilization that eliminates the residual negative effect of this reagent. Also, CSF2 increases blastocyst development together with the quality of the resultant blastocysts when added at D5 of culture. Moreover, MG132 during late maturation followed by 8 h IVF and CSF2 from D5 of culture improves the development of embryos and skews the blastocyst sex ratio towards female. This new approach of IVP may have important implications in the dairy cattle industry and opens up area for further investigation in human assisted reproduction.

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List of abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
AC	Adenylate cyclase
ADP	Adenosine diphosphate
AKT	Serine/threonine-specific protein kinase
AIK5	Activin like kinase 5
APC/Ccdh1	Anaphase promoting complex / cyclosome- cadherin 1
ATP	Adenosine triphosphate
AURKB	Aurora kinase B
BMPs	Bone morphogenic proteins
BSA	Bovine serum albumin
BUB1 and 3	Budding Uninhibited by Benzimidazoles 1 and 3
cAMP	Cyclic adenosine monophosphate
CCL2, CCL7	Chemokine C-C motif ligand 2 and 7
CCR	Chemokine C-C motif receptor
CDC20	Cell division cycle protein 20
CDC25B	Cell division cycle 25 B
CDK1	Cyclic dependent kinase 1
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
Chd	Chromatin decondensation
CLD	Cleaved
Co2	Carbon dioxide
COX2	Cyclooxygenase
CP	Catalytic core particle
Cr	Creatine
CREM	cAMP responsive element modulator
CSF arrest	Cytostatic factor arrest
CSF2	Colony stimulating factor 2
Ct	Threshold cycles
Cxs	Connexins
D	Day

DKK1	Dickkopf-1
Drp1	Dynamin related protein 1
DUB	Deubiquitination
E	Efficiency
E1	Ubiquitin activating
E2	Ubiquitin conjugating
E3	Ubiquitin ligating
ECM	Extracellular matrix
EGA	Embryonic gene activation
EGF	Epidermal growth factor
EPR2, EPR4	Prostanoid receptor of PGF2
Er Ca^{+}	Endoplasmic reticulum calcium
ERK	Extracellular single-regulated kinase
ERV	Endogenous retrovirus transposon
FCS	Fetal calf serum
FSH	Follicles stimulating hormone
GAGs	Glycosaminoglycans
GDFs	Growth differentiation factors
GDP	Guanosine diphosphate
GDPH	Glycerol-3-Phosphate dehydrogenase
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte macrophage- colony stimulating factor
Gs	G alpha subunit
GsP	G alpha subunit protein
GV	Germinal vesicle
GVBD	Germinal vesicle break down
h	Hour
HA	Hyaluronan
HAS2	Hyaluronan synthase type 2
ICSI	Intracytoplasmic sperm injection
IGF	Insulin growth factor
IL3,5	Interleukin 3, 5
INS	Inseminated
IRF7	Interferon regulatory factor 7
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation

IαI	Inter-alpha-trypsin inhibitor
iXCI	Imprinted X chromosome inactivation
LAP	Latency associated protein
LH	Luteinizing hormone
LTBP	Latent TGFB binding protein
M	Molar
MAD2	Mitotic arrest deficient 2
MADD	MAP kinase activating death domain
MAPK	Mitogen-activated protein kinase
MET	Maternal embryonic transition
mfn 1,2	Mitofusin 1,2
mg	Milligram
Mg+	Magnesium
MG132	N-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal
ml	Millilitre
mm	Millimetre
mM	Millimolar
MOS	Proto-oncogene, serine threonine kinase
MPF	Maturation or Mitosis or M-phase promoting factor
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MTZ	Maternal zygotic transition
NANOG	Homeobox protein (name based on Scottish legend Tír na nÓg)
ng	Nanogram
NOD2	Nucleotide binding oligomerization domain containing 2
NPR2	Natriuretic peptide receptor 2
NRF1	Nuclear respiratory factor 1
NT5E	5'-nucleotidase ecto
OPU	Ovum pick Up
Parkin	Parkin RBR E3 ubiquitin protein ligase
P4HB	Prolyl 4-hydroxylase beta-subunit
PBI	First polar body
PCr	Phosphocreatine
PCR	Polymerase chain reaction
PGC1	Peroxisome proliferative activated receptor, gamma, coactivator 1
PDEs	Phosphodiesterases
PGE2	Prostaglandin E2

PGR	Progesterone receptor
Pink	PTEN-induced kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PRKAR2B	Protein kinase cAMP-dependent type II regulatory subunit beta
Ptx3	Pentraxin 3
qPCR	Quantitative polymerase chain reaction
Rnf12	Ring finger protein 12
RIPK3	Receptor-interacting serine-threonine kinase 3
ROS	Reactive oxygen species
RP	Regulatory particle
rRNA	ribosomal ribonucleic acid
RTK	Receptor tyrosine kinase
rt-PCR	Reverse transcription polymerase chain reaction
SAC	Spindle assembly checkpoint proteins
sGC	Soluable guanylyl cyclase
SMAD	Small (SMA) and Mothers Against Decapentaplegic (MAD) proteins
SNP	Sodium nitroprusside
SOX2	Sex determining region Y-box 2
Sph	Sperm head
SRC	Proto-oncogene, non-receptor tyrosine kinase
TFAM	Transfer factor A
TGFB1	Transforming Growth Factor beta 1
TNFAIP6	Intrafollicularly synthesized tumor necrosis factor α-induced protein 6
tRNA	transfer ribonucleic Acid
TZPs	Trans zonal projections
UPS	Ubiquitin proteasome system
Wee1 / Myt1	Wee 1 like protein kinase / Myelin transcription factor 1
Xist	X-inactive specific transcript

Introduction

In vitro embryo production (IVP) is an important biotechnology in cattle breeding and with great potential to enhance the genetic merit of offspring for a range of commercially important traits including animal health (Camargo et al., 2006). There is great interest in using it in commercial herds accompanied with accurate genomic testing of donor females (Thomasen et al., 2016), or when combined *in vitro* culture with other biotechnologies such as ovum pick up (OPU) and artificial insemination (AI) (Merton, 2014 and Kaniyamattam et al., 2017).

In 2016, the largest number of IVP embryos transferred was in The Netherlands, with comparatively few transferred in the UK (**Figure 1A**). However, the major global players in IVP and transfer are to be found in Brazil and in the United States and Canada (**Figure 1B**).

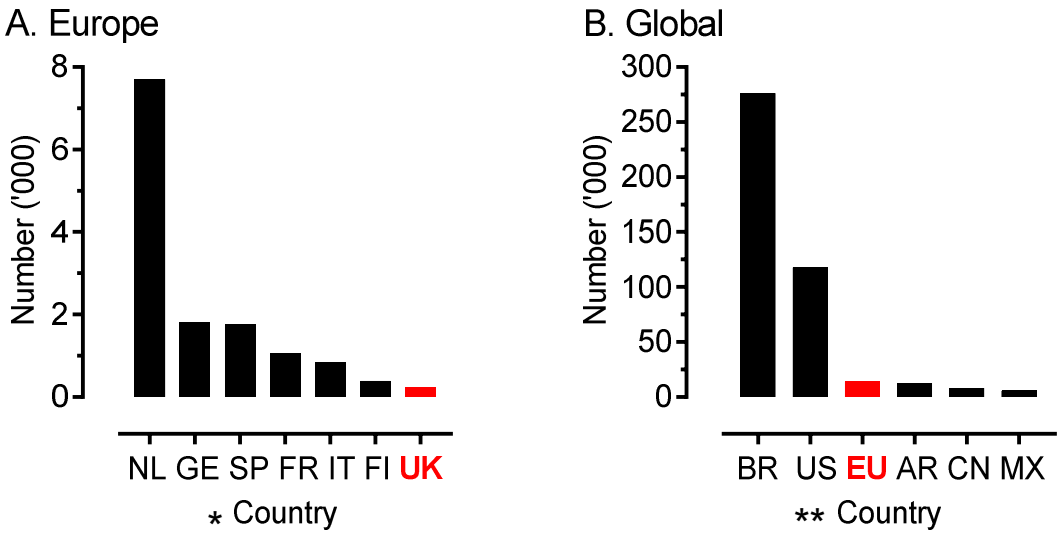

Figure 1. Number of *in vitro* embryos produced in different European countries (A), and globally in some countries worldwide (B) Source: Embryo Technology Newsletter 35, No. 4. IETS (2017).

* -NL=Netherland, GE=Germany, SP=Spain, FR=France, IT=Italy, FI=Finland and UK=United Kingdom

** -BR=Brazil, US=United States, EU=Europe, AR=Argentina, CN=Canada and MX=Mexico.

In Brazil, *in vitro* embryo production increased after 2003 and now represents around 59% of total world IVP production. This success is believed to be related to genetic aspects of Nelore cattle (*Bos indicus*) that lead to a larger number of antral follicles and oocytes that are more developmentally competent when cultured *in vitro* (Sartori et al., 2016) (**Figure 2**).

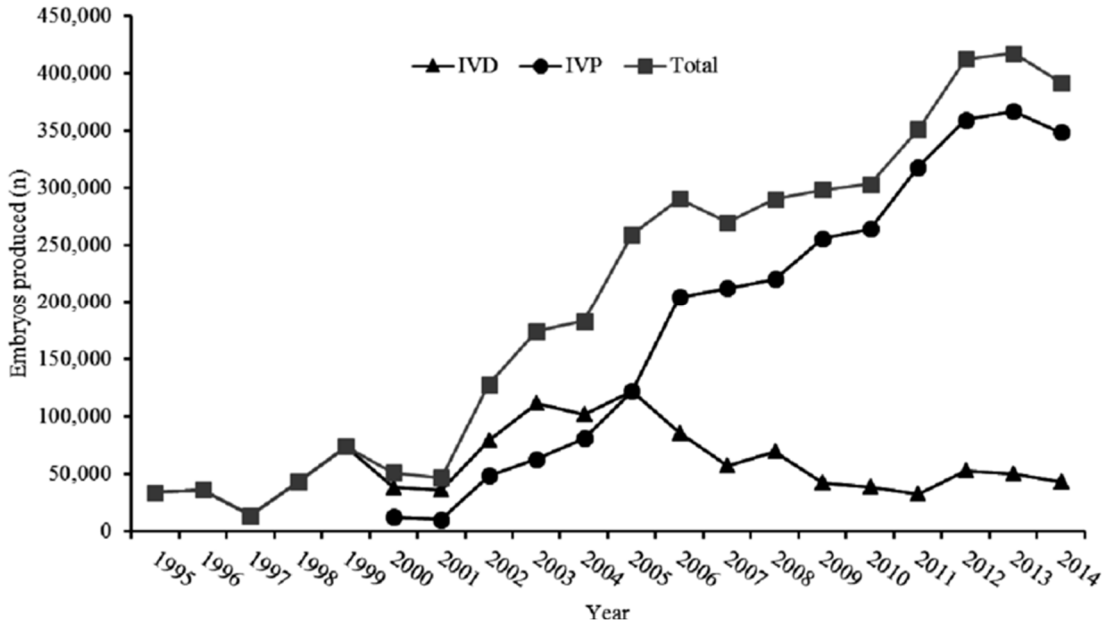

Figure 2. Number of embryos produced *in vitro* in Brazil between 1995 to 2014. (IVD = *in vivo* embryo derived, IVP = *in vitro* embryo production). Source: Sartori et al., 2016

There are three key steps to *in vitro* embryo production: *In vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* embryo culture (IVC) (Sirard and Coenen, 2006 and Lonergan and Fair, 2016). Despite years of research attempting to improve the efficiency of IVP in *Bos taurus* cattle, success rates are still low (Camargo et al., 2006). The situation is even poorer with the use of sex-sorted semen for IVP which aims to increase the proportion of female offspring born (Mikkola and Taponen, 2017).

In vitro maturation became the main concern in the research area of IVP, and could be alternative for conventional IVF to avoid ovarian hyperstimulation syndrome cases in women (Tannus et al., 2018 and Hatirnaz et al., 2018).

Recent studies have focused on the role of functional mitochondria during *in vitro* maturation of bovine oocytes and in maintaining optimal levels of mitochondrial DNA (mtDNA) and ATP; although what constitutes optimal is still poorly understood (Chiaratti et al., 2010; Gutnisky et al.,2013; Huang et al., 2014; Lee et al., 2014a and May-Panloup et al., 2016). Another approach that had been suggested to increase the efficiency of bovine IVP is shortening the incubation period of gametes during fertilization to avoid the incidence of polyspermy and accumulation of deleterious free radicals (Iwata et al., 2008; Enkhmaa et al., 2009 and Lopes et al., 2010). Moreover, in the last decade, there has been a great deal of interest in the idea of supporting and nourishing embryos beyond morulation through utilizing different growth factors and cytokines (Neira et al., 2010; Hansen et al., 2014; Moreno et al. 2015 and Tribulo et al., 2018).

Therefore, the purpose of the current study was to enhance the proportion and quality of blastocysts by targeting mitochondrial activity during oocyte maturation and through nourishing embryos with cytokines beyond morulation. Also, monitoring the possibility of shifting the sex ratio of the resultant blastocysts towards females in a low cost and efficient *in vitro* embryo production system.

1. Literature review

1.1. Factors influencing developmental competence

Developmental competence is defined as the ability of gametes to mature, fertilize and develop beyond the stage of embryonic gene activation (Kempisty et al., 2015). However, Gilbert et al. (2015) described that both developmental competence of oocytes and quality of embryos are closely related and that the proportion of embryos reaching the blastocyst stage is not the only metric for the improvement of *in vitro* embryo production. Other, less well characterised factors, such as chromosomal number, can influence subsequent development (Viuff et al., 2000). Numerous factors affect the developmental competence of bovine oocytes and quality of embryos cultured *in vitro*. Although delays in cytoplasmic maturation can lead to low developmental competence of matured oocytes *in vitro*, embryo culture conditions determine cell cycle length, number of zygotes reaching blastocysts, quality and primary sex ratio of the resultant blastocysts.

Most factors that negatively affect the developmental competence of oocytes are related to defects in cytoplasmic maturation (Damiani et al., 1996; Watson, 2007; Demyda-Peyras et al., 2013; Kim et al., 2018 and Rybska et al., 2018). Moreover, the origin of oocyte plays an important role in determining the degree of cytoplasmic maturation. Therefore, *in vivo* retrieved oocytes by ovum pick up (OPU) can lead to greater post fertilization development than *in vitro* collected oocytes from abattoir derived ovaries (Blondin et al., 2002 and Merton et al., 2003). On the other hand, preservation status of ovaries before aspiration, maturation media, follicular fluid composition, temperature and vascularity plays an important role in the acquisition of developmental competence of oocytes (Yang et al., 1990; Sutton et al., 2003; Sinclair et al., 2008 and Hung et al., 2015). Also, parity affects post-fertilization development and *in vitro* retrieved bovine oocytes; where multiparous cows yield more blastocysts than heifers (Rizos et al., 2005).

There is increasing interest in the role of functional mitochondria in oocytes during maturation *in vitro* (Tarazona et al., 2006; Van Blerkom, 2008; Gutnisky et al., 2013; Huang et al., 2014; Lee et al., 2014a and May-Panloup et al., 2016). Also, the importance of maintaining certain levels of mitochondrial DNA (mtDNA) in the oocyte that provides adequate Adenosine Tri-Phosphate (ATP) and enables embryos to develop during the preimplantation period (Chiaratti et al., 2010).

While, nitric oxide increases mitochondrial DNA replication (St John et al., 2014), the nitric oxide donor sodium nitroprusside (SNP) improves the development of bovine embryos cultured *in vitro* (Viana et al., 2007). Moreover, proteasomal machinery might determine the developmental competence of oocytes *in vitro*, were MG132 (a proteasome inhibitor) may salvage important proteins related to glycolysis and apoptosis in bovine oocytes (You et al., 2012a). Furthermore, MG132 participates in the reduction of mitochondrial degradation or mitophagy during late maturation by increasing mitofusin (Mfn) proteins that are related to mitochondrial fusion which is an essential process in the development of mouse embryos (Chen et al., 2003 and Tanaka et al., 2010). On the other hand, fertilization events can determine the subsequent development of embryos cultured *in vitro*. Therefore, incubation period of gametes for fertilization affects the development of zygotes reaching the blastocyst stage together with the sex ratio of the resultant embryos (Rehman et al., 1994; Ward et al., 2002; Kochhar et al., 2003; Nedambale et al., 2006 and Sattar et al., 2011). Moreover, long incubation periods are associated with increased incidences of polyspermy and accumulation of deleterious free radicals (Sumantri et al., 1997; Kochhar et al., 2003 and Iwata et al., 2008; Enkhmaa et al., 2009 and Lopes et al., 2010).

In terms of embryo culture, suboptimal conditions affect cell cycle length and blocks bovine embryonic development *in vitro* at around 8-16 cell stage (Holm et al., 1998 and Carambula et al., 2009). Moreover, in sex sorted semen, culture media composition can

skew the sex of developing embryos in preference of females (Siqueira and Hansen, 2016). However, it has been reported that male embryos developed more rapidly than female embryos *in vitro* (Xu et al., 1992; Dobbs et al., 2013a and Green et al., 2016). Also, the *in vitro* environment can lead to epigenetic modifications and skew the sex ratio in preference of male embryos in mammals (Tan et al., 2016). Recently, the role of cytokines in multiple cellular process, including proliferation, differentiation, cell cycle regulation and apoptosis during preimplantation period, has attracted attention due to their importance in the salvage of embryos cultured in vitro (Neira et al., 2010; Hansen et al., 2014; Moreno et al. 2015 and Tribulo et al., 2018).

In spite of the different approaches to increase the developmental competence of oocytes and quality of bovine embryos, numerous factors might explain variabilities of *in vitro* embryo production outcomes (Baruselli et al., 2016). Therefore, the aims of the current study were firstly to investigate the effects of novel combinations of the nitric oxide donor Sodium Nitroprusside (SNP) and proteasomes inhibitor MG132 (N-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal) during IVM on mitochondrial activity during oocyte maturation, and on post-fertilization development in bovine embryos. Secondly, to investigate the effects of novel combinations of Transforming Growth Factor-B1 (TGFB1) and Granulocyte Macrophage- Colony Stimulating Factor (GM-CSF) administered at D5 during *in vitro* culture on embryonic development and blastocyst yield, and quality of bovine embryos at D8 of IVC. Finally, to determine the best combinations of these approaches in order to develop an improved system for bovine IVP.

1.2 Oocyte Meiotic arrest and resumption

Oocytes in secondary follicles are arrested at germinal vesicle (GV) stage of development, achieved by high levels of cyclic Adenosine monophosphate (cAMP) inside oocytes and cumulus cells (Albuz et al., 2010 and Celik, 2015). Hypoxanthine and

adenosine are among those components in follicular fluid that inhibit phosphodiesterase (PDE) enzyme activity which, in turn, inhibits the conversion of ATP to cAMP. Also, PDE is inhibited by elevated levels of cyclic guanosine monophosphate (cGMP) that diffuses into oocytes from cumulus cells through membrane protein gap junction Connexins (Cxs) (Shimada, 2012 and Shuhaibar et al., 2015) (**Figure 1.1**).

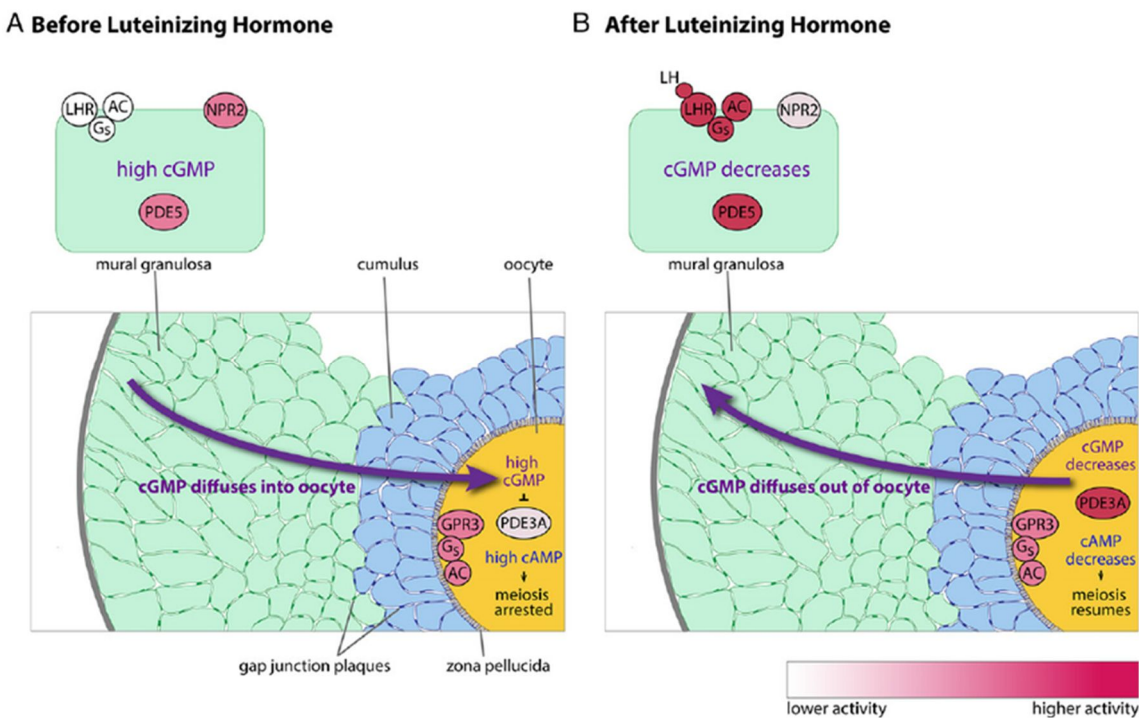

Figure 1.1. Schematic illustration of the effect of gap junctions between cumulus cells and oocytes on cAMP and cGMP transfer during meiotic arrest and resumption. Note that luteinizing hormone triggers the resumption of meiosis by decreasing cAMP and cGMP in the oocyte. Source: Shuhaibar et al. (2015).

Activation of protein kinase A (PKA) as a response to the elevation of cAMP in oocyte, leads to phosphorylation of cyclic dependent kinase1 (CDK1) and inactivation of maturation promoting factor through inactivation of cell division cycle 25 B phosphatase (CDC25B) and the activation of Wee 1 like protein kinase/Myelin transcription factor 1 (Wee 1/Myt 1 kinase), which in turn inhibits the resumption of meiosis in mammalian oocytes (Potapova et al., 2009 and Tripathi et al., 2010). In pre-ovulatory follicles, oocytes resume meiosis during the luteinizing hormone (LH) surge, and this releases the

oocyte from diplotene arrest by indirect action on cumulus cells (Tripathi et al., 2010 and Tiwari et al., 2018). However, in secondary follicles, bovine oocytes resume meiosis spontaneously following removal and *in vitro* culture (Aktas et al., 2003). The stimulatory hormones and growth factors in maturation medium play an important role in nuclear and cytoplasmic maturation of cattle oocytes (Ahumada et al., 2013 and Xiao et al., 2014). LH activates G alpha subunit (Gs) and other G protein receptors by mechanisms that are not fully understood, but involve dephosphorylate transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2), also known as guanylyl cyclase-B, which reduces the production of cGMP. Moreover, reduction in cGMP in cumulus cells and diffusion of this metabolite from oocytes to cumulus cells, relieves the inhibitory effect of cGMP on PDEs and resumption of meiosis in mouse and rat oocytes (Egbert et al., 2014 and Shuhaibar et al., 2015).

Although bidirectional crosstalk between oocytes and cumulus cells by gap junctions provides essential cAMP for meiotic arrest, meiosis is triggered when oocytes are removed from follicular fluid. Resumption of meiosis is activated by M-phase promoting factor (MPF) which has two waves of elevation during *in vitro* maturation of bovine oocytes (i.e. during metaphase-I and II) (Wehrend and Meinecke, 2001). Moreover, resumption of meiosis requires abundance of cyclin B, which elevates and stabilizes MPF levels (Levesque and Sirard, 1996 and Tiwari and Chaube, 2017). In addition, elevation of mitogen activated protein kinase (MAPK) occurs simultaneously with MPF in bovine oocytes during *in vitro* maturation and has an important role in mediating metaphase II (MII) arrest and maintenance of elevated levels of MPF at that stage (Wehrend and Meinecke, 2001; Gordo et al., 2001 and Prochazka and blaha, 2015).

Several factors are provided through gap junctions including cAMP and RNA that are essential for cytoplasmic maturation during meiotic arrest (Thomas et al., 2004 and Macaulay et al., 2014, 2016). Also, trans-zonal projections (TZPs) or gap junctions

remain in contact with the oolema during the first 9 h of maturation in bovine. However, their detachment continues until 22 h of maturation when the connection is completely lost forming the perivitelline line space (Macaulay et al., 2014).

1.3 Nuclear and cytoplasmic maturation

Oocytes undergo various stages of development after removal from follicles *in vitro*. These include changes in nuclear and cytoplasmic maturation. After aspiration and *in vitro* maturation, subsequent nuclear events take place in oocytes which include: germinal vesicle breakdown (GVBD) within (6.6-8 h), condensation of chromosomes (8-10.3 h), metaphase I spindle formation, separation of the homologous chromosomes and extrusion of first polar body (PBI), and arrest at metaphase II (18-24 h) *in vitro* (Sirard et al, 1989). On the other hand, polar body extrusion during *in vitro* maturation of oocytes occurs after 12 h, with maximum peak in the rate of PBI between 16-20 h in the bovine. Also, cleavage and development of zygotes to the blastocyst stage was greater for oocytes matured for 16-20 h than those matured for 22-24 h (Van der Westerlaken et al., 1994). On the other hand, cytoplasmic maturation includes changes in the content and structure of molecules and organelles within the cytoplasm. During maturation, cumulus cells are responsible for the provision of oocytes with cAMP, cGMP, mRNA and proteins via gap junctions between cumulus cells and oocytes (Eppig, 1991; Macaulay et al., 2014 and Biase et al., 2018). It has been hypothesized that follicle stimulating hormone (FSH) affects communication between cumulus cells and oocytes, and plays a key role in the establishment of oocyte competence in the bovine (Li et al., 2012 and Sugimura et al., 2017) (**Figure 1.2**). Also, elevation of MPF is essential to release oocyte from diplotene arrest to metaphase (Wu et al., 1997 and Jones, 2004 and Tiwari et al., 2018). Moreover, MPF stabilized by MAPK play a crucial role after meiosis resumption to MII arrest (Gordo et al., 2001 and Tian et al., 2002). Whereas, a decline of MAPK after fertilization, initiated by increase Ca^{+} oscillation, is essential for pronuclear formation in the mouse zygote

(Gonzalez-Garcia et al., 2014). However, MAPKs along with Aurora kinase B (AURKB) are considered important elements for proper development of mouse embryo (Xu et al., 2012).

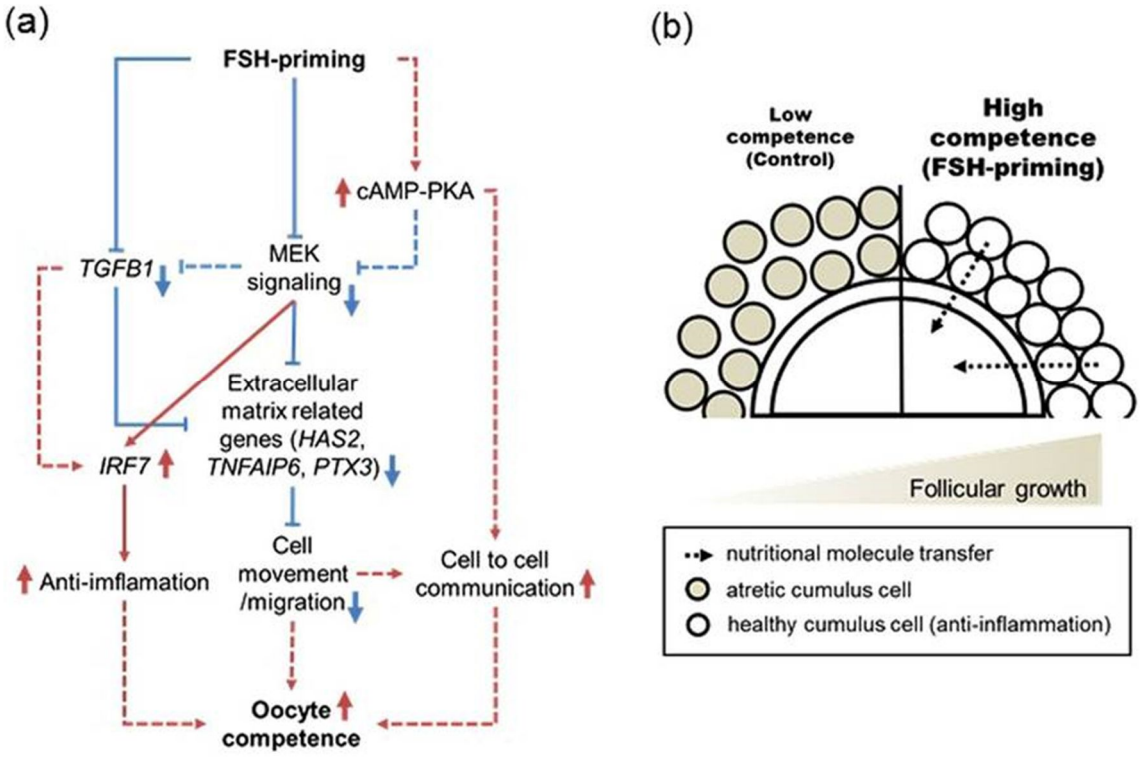

Figure 1.2. Hypothetical diagram of the effect of FSH on cumulus cells and the developmental competence of bovine oocytes. Note that healthy cumulus cells with restricted movement are nourished oocytes with the required nutrient during maturation. Source: Sugimura et al. (2017).

With regards to structural changes, Golgi apparatus is responsible for the production of cortical granules, which translocate from the centre to the plasma membrane adjacent to the zona pellucida as oocytes progress to metaphase-I (Liu, 2011 and Racedo et al., 2012). While mitochondria affect cell fate during development, they change their location and shape during maturation depending on follicular size. They locate in the centre of oocytes and are rounded in shape in follicles less than 100 µm and hooded in the periphery in oocytes greater than 110 µm (Fair, 1995). The phenotypic state of hooded shaped mitochondria, with reduction in number of cristae and increasing electron density

in their matrix, persist in cattle to the 8-16 cell stage. This phenomenon has led researchers to measure ATP content in oocytes and early embryos (Scantland et al., 2014).

1.4 Mitochondrial activity

In recent years, there has been increasing interest in the effect of functional mitochondria on embryonic development *in vitro*. Mitochondria are organelles inherited from the maternal line (applies to > 99% in mammals) which play a vital role in cell function and are considered to be indicators for oocyte competence (Van Blerkom, 2011). In addition to roles in Ca^{+} homeostasis, they are the main source of energy that produce adequate ATP by metabolism of pyruvate through oxidative phosphorylation via the electron transport chain within mitochondrial cristae (Van Blerkom, 2008; Wang et al., 2009 and Dalton et al., 2013). However, Scantland et al. (2014) reported that both glycolysis and oxidative phosphorylation is limited in oocytes during maturation and in early bovine embryos. Also, the adenosine salvage pathway, which involves the degradation of cAMP to AMP by phosphodiesterases and then to ADP (reaction 1) in the presence of adenylate kinase, followed by conversion of ADP to ATP and creatine (Cr) during hydrolysis of phosphocreatine (PCr) by creatine kinase (reaction 2), might be an alternative ATP production pathway during bovine oocyte maturation and early embryo development (**Figure 1.3**).

$$\mathrm{AMP} + \mathrm{ATP} \leftrightarrow 2\,\mathrm{ADP} \qquad (1)$$

$$2\,\mathrm{ADP} + 2\,\mathrm{PCr} \leftrightarrow 2\,\mathrm{Cr} + 2\,\mathrm{ATP} \qquad (2)$$

Figure 1.3. Proposed adenosine salvage pathway for production of ATP during oocyte maturation and early bovine embryo development. Source: Scantland et al. (2014).

ATP levels in oocytes during different stages of maturation depends on cellular ATP demand and consumption (Dalton et al., 2014). Therefore, ATP content is increased within oocytes that resume meiosis after GV stage in the bovine (Nagano et al., 2006 and Stojkovic et al., 2001). Before maturation, mean ATP content in bovine oocytes that have brown ooplasm and a dark zone on the periphery or dark clusters is 0.66 ± 0.3 pmol/oocyte and 0.53 ± 0.2 pmol/oocyte respectively, with a polar body extrusion rate 81% and 85% respectively after maturation. Also, ATP content in these groups after maturation is 0.88 ± 0.17 pmol/oocyte and 1.00 ± 0.26 pmol/oocyte respectively. Moreover, ATP content 12 h prior to maturation varies (0.40 to 0.70 pmol/oocyte), then sharply increases between 12 h -17 h (0.60 to 1.00 pmol/oocyte), and increases further between 17 h - 22 h (1.20 pmol/oocyte) (Nagano et al., 2006). Stojkovic et al. (2001) reported that ATP content and developmental ability of oocytes that have multiple layers of cumulus cells is higher than denuded oocytes. Moreover, they demonstrated that there is no difference between oocytes which extrude their first polar body and those that did not in terms of ATP content at the end of maturation.

Mitochondria have the ability to balance ATP supply and demand that is critical for oocyte competence and affects fertilization outcomes (Van Blerkom, 2011). Also, it has been reported that decline of mitochondrial function by reduction of ATP content in mature oocytes is an ageing-associated marker in cattle (Iwata et al., 2011), mice and hamster (Simsek-Duran et al., 2013). ATP production is crucial to maintain resting levels of Ca^{+} in matured oocytes and to sustain Ca^{+} oscillations at fertilization (Darbandi et al., 2016). Moreover, insufficient supply of ATP drives stored endoplasmic reticulum (Er) Ca^{+} release. If extended then this results in failure of Er Ca^{+} refill that leads to post fertilization defects (Takahashi et al., 2000; Liu et al., 2001; Gordo et al., 2002 and Igarashi et al., 2005). On the other hand, high levels of ATP at MII is associated with lower developmental ability in bovine oocytes (Nagano et al., 2006). Also, increased ATP levels are associated with reduction of Mg^{+} metabolites due to high affinity of ATP to chelate

Mg^{+} that results in reduction of protein synthesis and cell growth (Pontes et al., 2015). Moreover, increased ATP production generated high reactive oxygen species that associated with mitochondrial DNA damage (Fernandez-Silva et al., 2003), also it change mitochondrial dynamics by decreasing fission rates and elongation of mitochondria (Liesa et al., 2013).

Mitochondria are the only organelles in eukaryotes that have DNA in their matrix. This DNA encodes 13 proteins for oxidative phosphorylation in most mammalians species and 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs) and has one non-coding region or control region, the D-loop (Appendix-I). Mitochondrial function depends on the communication between nuclear DNA and several copies of DNA contained within the mitochondrion (Chiaratti et al., 2010 and Zhang et al., 2017). St John (2014) reviewed an increase in mitochondrial copy number during mammalian oogenesis up to metaphase-II that then undergoes a significant reduction up to the blastocyst stage. Also, Santos et al. (2006) demonstrated the importance of mtDNA copy number in human oocytes in determining fertilization outcome. Moreover, copy number is reduced in bovine oocytes with maternal age, which can negatively affect fertilization outcome (Iwata et al., 2011 and Takeo et al., 2013). In contrast, Cree et al. (2015) did not find an age-related effect on mtDNA copy number in cattle. Munakata et al. (2016) demonstrated the relationship between cumulus cells layers surrounding oocytes, lipid content, mtDNA copy number and ATP content in porcine. Recently, addition of external mtDNA to oocyte increased the copy number in the resultant blastocysts and might enhance post implantation development of bovine embryo (Srirattana and St John, 2017, 2018).

The correlation between mtDNA copy number and ATP content is controversial. May-Panloup et al. (2007) found no correlation between mtDNA copy number and ATP content. However, Jeng et al. (2008) demonstrated a positive correlation between ATP production and mitochondrial respiratory enzymatic activity which increased with

increase mtDNA copy number. In contrast, Iwata et al. (2011) showed a negative correlation between mtDNA copy number and ATP content in bovine oocytes.

Different phenomenon relates to mitochondrial activity including fusion, fission and mitophagy (Youle and Van der Bliek, 2012). Also, the balance between fusion and fission determines the length of mitochondria and the degree of close network they formed in their cytoplasm (Westermann, 2010). Moreover, phosphorylation of dynamin related protein (DRP1) by different kinases plays a key role in the initiation of mitochondrial fission (Van der Bliek et al., 2013 and Udagawa et al., 2014). On the other hand, mitochondrial fusion depends on the abundance of mitochondrial outer membrane proteins or Mitfusin-1 and 2 (Mfn1, 2), that is an essential process for embryonic development (Chen et al., 2003; Wakai et al., 2014 and Zhang et al., 2016). However, degradation of mitofusins lead to the segregation of dysfunctional mitochondria that precedes mitochondrial degradation or mitophagy (Chan et al., 2011).

Lee et al. (2014a) reported that metabolic activity of mitochondria is crucial for oocyte maturation, and both mitochondrial metabolic activity and DNA replication contribute in the development competence of porcine oocytes. Mitochondrial DNA replication passes through a complex series of biological events, and intrinsic nitric oxide is a key promoter in the process of mtDNA replication. Moreover, nitric oxide enhances mitochondrial biogenesis and produces functional mitochondria that are able to generate adequate ATP through oxidative phosphorylation in myocytes (Nisoli et al., 2004 and Tengan et al., 2012). However, the effect of external nitric oxide donor on mitochondrial DNA copy number in bovine oocyte is poorly studied.

There is variability in the determination of mtDNA copy number in cattle. May-Panloup et al. (2005); Iwata et al. (2011) and Cree et al. (2015) reported a range of 3 to 6 x 10^5 copies of mtDNA in bovine oocytes. However, Srirattana et al. (2017) demonstrated that copy number for *Bos indicus* and *Bos taurus* breed is between 1.9 to 2.8 x 10^5. Whereas the development of bovine oocytes is not compromised until mtDNA copy number

reaches 5 x 10^4 copy (Chiaratti et al., 2010). Also, the treatment of oocytes from young but not aged cows with 10 μM MG132 increases mtDNA copy number (Kansaku et al. 2017). Moreover, Sato et al. (2014) and Itami et al. (2015) reported an increasing in copy number after treatment of porcine oocytes with MG132.

1.5 Proteasomal degradation

Maintaining intracellular proteins by synthesis and degradation is a continuous process regulated by lysosomal and proteasomal activity (Martinez-Vicente et al., 2005). However, metabolic balance between protein synthesis and degradation is achieved by proteasomal but not the lysosomal system (Rothman, 2010). The proteasome is composed of two complexes: a catalytic core particle (CP), or so called 20s proteasome, and two or one terminal of regulatory particles (RP), also called 19s proteasome. Binding 19s RP to both or one ends of 20s CP forms an enzymatically active proteasome (Ciechanover, 2005; Tanaka, 2009). Ubiquitination by ubiquitin, a small chaperone protein that links with target proteins by isopeptide chains, is required to mark protein substrate for degradation by the proteasome (Nagyova et al., 2018). Among distinguished ubiquitin enzymes are: E1 (Ubiquitin activating), E2 (Ubiquitin conjugating), and E3 (Ubiquitin ligating) (Saez and Vilchez, 2014 and Vilchez et al., 2014) (**Figure 1.4**). Ubiquitination of proteins is a reversible process undertaken by deubiquitinating enzymes (DUBs). Also, the ubiquitin- proteasome system (UPS) mediates a variety of cellular functions including cell growth, differentiation, stress, cell death and embryogenesis (Varshavsky, 2005 and Higuchi et al., 2018). Proteasomal degradation is an ATP dependant process where ATP is essential for the 19s regulatory particle and the ubiquitination reaction. Also, the activity of the proteasome is suppressed by low ATP supply or even when ATP is at physiological levels (Huang et al., 2010). On the other hand, proteasomal degradation is an essential process that mediates meiotic events,

sperm oocyte interactions, sperm mitophagy or degradation of paternal mitochondria directly post insemination (Karabinova et al., 2011 and Song et al., 2016).

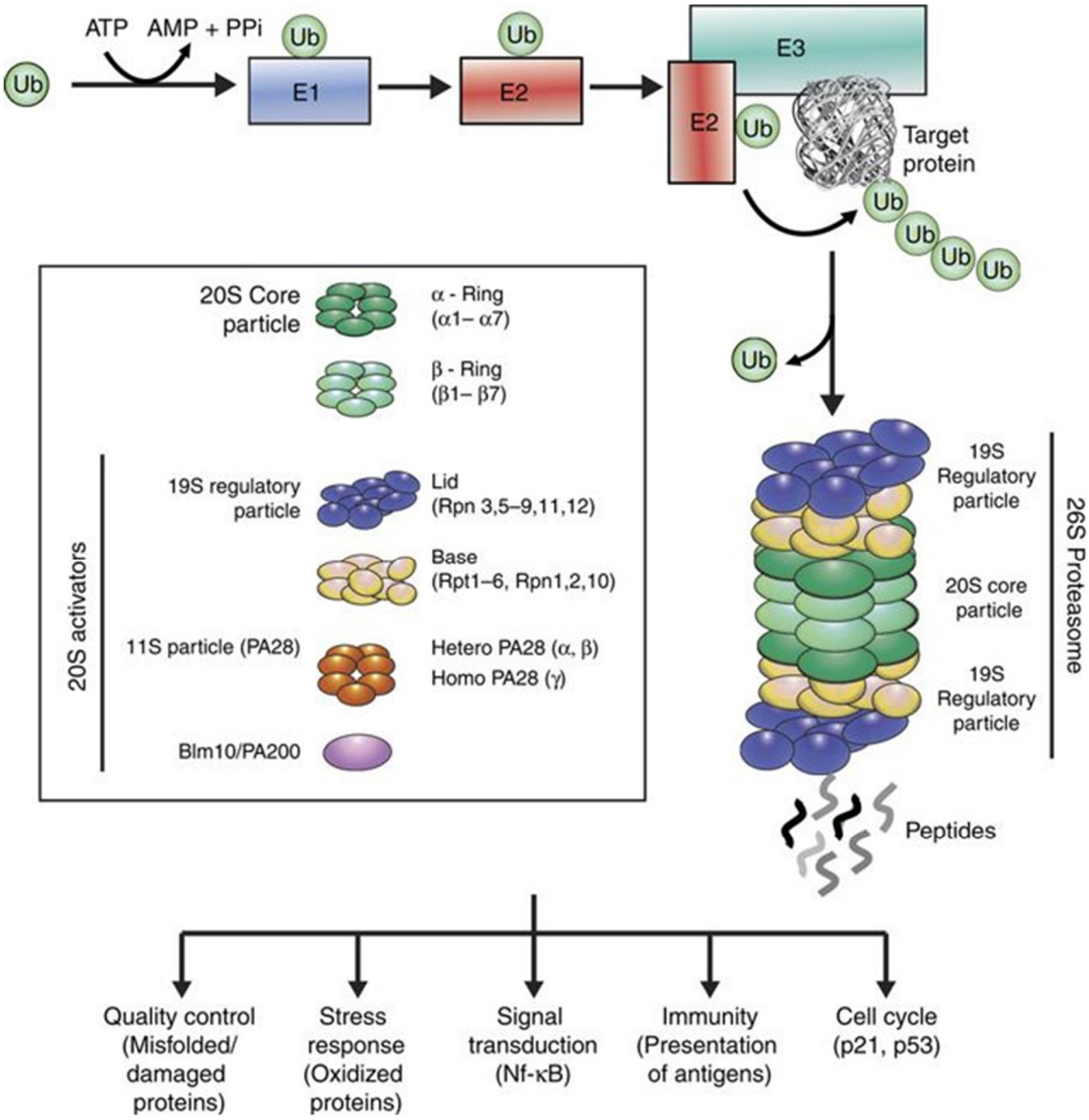

Figure 1.4. Schematic diagram of the proteasomal component and degradation of proteins by the proteasome that regulate many biological functions. Ubiquitination reaction is an ATP dependent process. Source: Vilchez et al. (2014).

MG132 is a cell permeable peptide aldehyde which inhibits the activity of chymotrypsin like activity of the 26S proteasomal core. This in turn inhibits cumulus cell expansion and delays the breakdown of TZPs or gap junction closure if continued during maturation the resumption of meiosis (Nagyova, 2018). However, a short period of exposure to MG132 during late oocyte maturation enhances the development and viability of transferred

embryos in cattle (You et al., 2012a), yak (Xiao et al, 2014), pig (You et al., 2012 and Shen et al., 2017) and rat (Ono et al., 2011). The precise kinetics of the effect of MG132 during maturation on embryonic development is not fully understood. However, MG132 inclusion during late *in vitro* maturation of bovine oocytes resulted in reduction of proteasomal degradation that led to upregulation of numerous proteins in oocytes, including the glycolytic protein glycerol-3-phosphate dehydrogenase (GDPH) and antiapoptotic protein beta-subunit of prolyl 4-hydroxylase (P4HB), which have beneficial effects on the development of bovine embryos (You et al., 2012a).

1.6 Metaphase-Anaphase and first polar body extrusion

Different mechanisms regulate mRNA translation in oocytes and these play an important role in the determination of oocyte maturation and meiotic events (Susor et al., 2015; Sousa-Martins et al., 2016 and Kalous et al., 2018). During prophase and prometaphase, anaphase promoting complex/cyclosome- cadherin 1 (APC/C^{Cdh1} complex) specifically target the degradation of cell division cycle protein 20 (CDC20) when cyclin-dependent kinase 1 (CDK1) is low (Reis et al., 2007 and Nabti et al., 2014). The reaction is then switched off to activate APC/C^{CDC20} complex when high activity of CDK1 occurs. CDK1 and mammalian target of rapamycin (mTOR) regulate the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) that promotes translation during meiosis in support of spindle assembly (Mayer et al., 2014 and Jansova et al., 2017). CDC20 alongside with spindle assembly checkpoint proteins (SAC) is an important cell cycle division regulator that transfers the cell from metaphase to anaphase. Also, CDC20 activates ubiquitin E3 ligase APC/C which in turn activates ubiquitin securin and cyclin B1 (Holt and Jones, 2009 and Karabinova et al., 2011). Moreover, after ubiquitination and degradation of securin, separase cleaves cohesin (complex linking chromatin) that initiates chromatin separation (Terret et al, 2003 and Singleton et al., 2017) (**Figure 1.5**). To prevent premature start of anaphase, mitotic arrest deficient 2 (MAD2), budding

uninhibited by benzimidazoles 1 (BUB1) and BUB3 proteins together with CDC20 form a checkpoint complex that inhibits the formation of the APC/C^{CDC20} complex. Downregulation of cell division cycle protein 20 (CDC20) impairs spindle assembly, chromosomal segregation and extrusion of the first polar body in cattle (Yang et al., 2014). On the other hand, proteasomal degradation of cyclin B1 is an important process for the cell to exit mitosis and separation of sister chromatids (Holloway et al., 1993; Josefsberg et al., 2000; Peters, 2002; Chang et al., 2003; Herbert et al., 2003 and Parry et al., 2003).

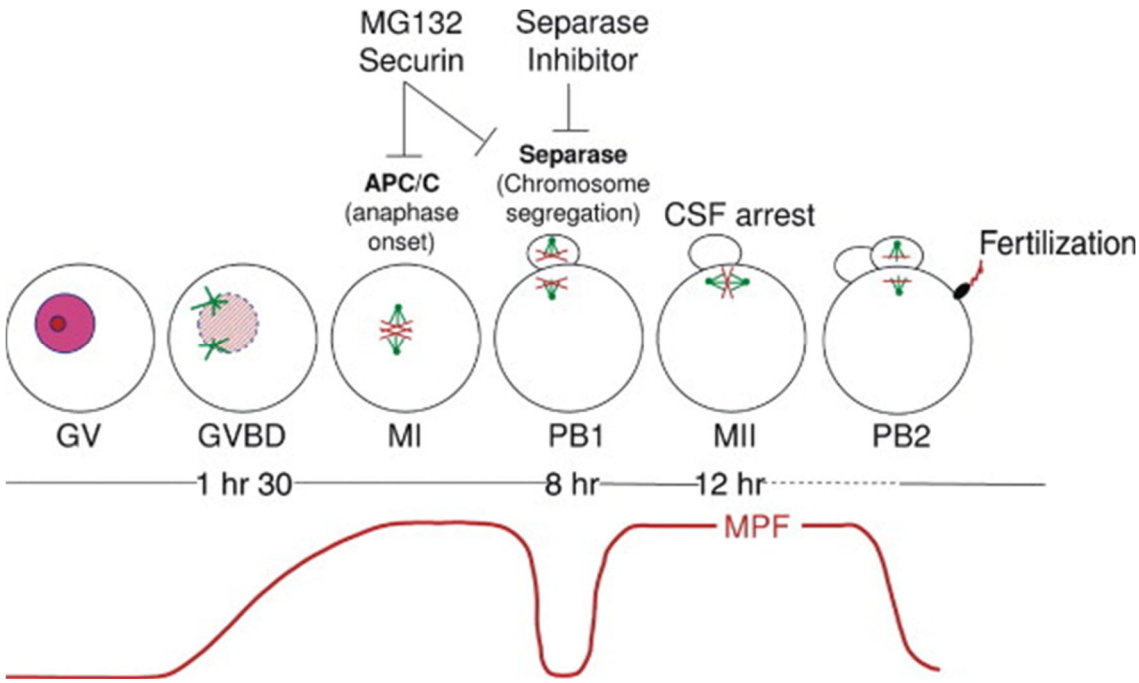

Figure 1.5. Schematic diagram of the effect of MG132 and separase inhibitor during transition and exit from metaphase I in the mouse oocyte. Note that presence of MG132 inhibit chromosome segregation during maturation and cytostatic factor (CSF) activity in MII arrest. Source: Terret et al. (2003).

The proteasomes inhibitor MG132 maintains high levels of cyclin B1 in rat oocytes that result in incomplete chromosomal segregation. Also, addition of MG132 at any stage during oocyte maturation in rats, even just before emission, prevents polar body extrusion (Josefsberg et al., 2000). However, in contrast to the rat, porcine oocytes show little sensitivity to MG132 treatment and sustain their development beyond metaphase I

even with the presence of a higher concentration than 10 μM of MG132 during *in vitro* maturation (Chmelikova et al., 2004). Moreover, administration of MG132 during late maturation had no effect on nuclear maturation of oocytes in cattle (You et al., 2012a), and Yak (Xiao et al, 2014).

Little is known regarding the fate of the first polar body in cattle. However, Hyttel et al. (1986) demonstrated the degeneration of first polar body between 30-40 h of maturation in some bovine oocytes cultured *in vitro*. In humans, Schmeirer and Wessel (2011) reported that apoptosis of first polar body of human oocyte occurs 17-24 h after its formation and that the degraded fragment remained trapped within the zona pellucida. The kinetics of polar body degradation is not established in the cow, however, in the mouse, it was reported that the proto-oncogene serine threonine kinase/Mitogen activated protein kinase (Mos/MAPK) pathway has a key role in regulation of first polar body degradation (Choi et al., 1996).

1.7 Cumulus cells and extracellular matrix

It was discussed earlier (Sections 1.2 and 1.3) that crosstalk and nourishing relationships between oocytes and cumulus cells during *in vitro* maturation occurs by transferring small metabolites through gap junctions or the trans zonal projections (TZPs). The beneficial effect of gap junctions and cumulus cells depends on the maturation status of the oocyte and the method of fertilization that subsequently affects the development of mouse embryos (Zhou et al., 2016). During *in vitro* fertilization, the presence of cumulus cells plays an important role in trapping spermatozoa or guiding them to the oocyte for fertilization (Van Soom et al., 2002; Sun et al., 2005 and Oren-Benaroya et al., 2008). Moreover, cumulus cells create an environment around the oocyte that affects the fertilizing ability of spermatozoa (Tanghe et al., 2003; and Hong et al., 2009). The average number of cumulus cells that surrounds a single matured bovine oocyte is around 2.1 x 10^4 cell (Hashimoto et al., 1998). Also, the density of cumulus cells

surrounding the oocyte depends on oocyte quality where the matured human oocyte has more cumulus cells than atretic or degenerated oocytes (Lourenco et al., 2014). Fertilization is affected by the composition of the extracellular matrix (ECM) of expanded cumulus cells that depends on the relationship between the secretion of the glycosaminoglycan hyaluronan (HA) through degradation of glucose in the hexosamine biosynthesis pathway, and the HA-associated ECM proteins that including hyaluronan synthase type 2 (HAS2); inter-alpha-trypsin inhibitor (IαI); intrafollicularly synthesized tumor necrosis factor α-induced protein 6 (TNFAIP6) and pentraxin 3 (Ptx3) (Gutnisky et al., 2007 and Nagyova et al., 2012).

In human, HA treatment favours the selection of mature sperm that have good DNA integrity, which in turn improves fertilization and embryo development (Parmegiani et al., 2010 and Worrilow et al., 2013). Moreover, pre-treatment of human sperm with cumulus oophorus increases the binding capacity of their zona (Franken and Bastiaan, 2009). Furthermore, utilizing Cumulus Oocyte Complexes (COCs) for the selection of human spermatozoa increases the chance of the development of embryos produced by ICSI (Intracytoplasmic sperm injection) and resulted in high blastocyst yield (Wang et al., 2018). On the other hand, recently, Niringiyumukiza et al. (2018) reviewed the involvement of prostaglandin E2 (PGE2, a potent inflammatory mediator) in the reduction of ECM viscosity in cumulus cells that facilitated sperm penetration, protected sperm from the phagocytic activity of neutrophils, and increased sperm motility, binding, survival and function that led to successful fertilization (**Figure 1.6**).

It has been reported that cAMP increases the survival of cumulus cells and stabilizes the cumulus matrix that results in increased fertilizability of mouse oocytes cultured *in vitro* (Giacomo et al., 2016). On the other hand, administration of MG132 during *in vitro* maturation of porcine oocytes inhibits the essential covalent link between heavy chain IaI and HA, and reduces the expression of HA-associated proteins (Yi et al., 2008 and Nagyova et al., 2012). In addition, the disruption of proteasomal activity by proteasomal

inhibitors at the time of insemination led to fertilization defects in human and bovine zygotes (Rawe et al., 2008).

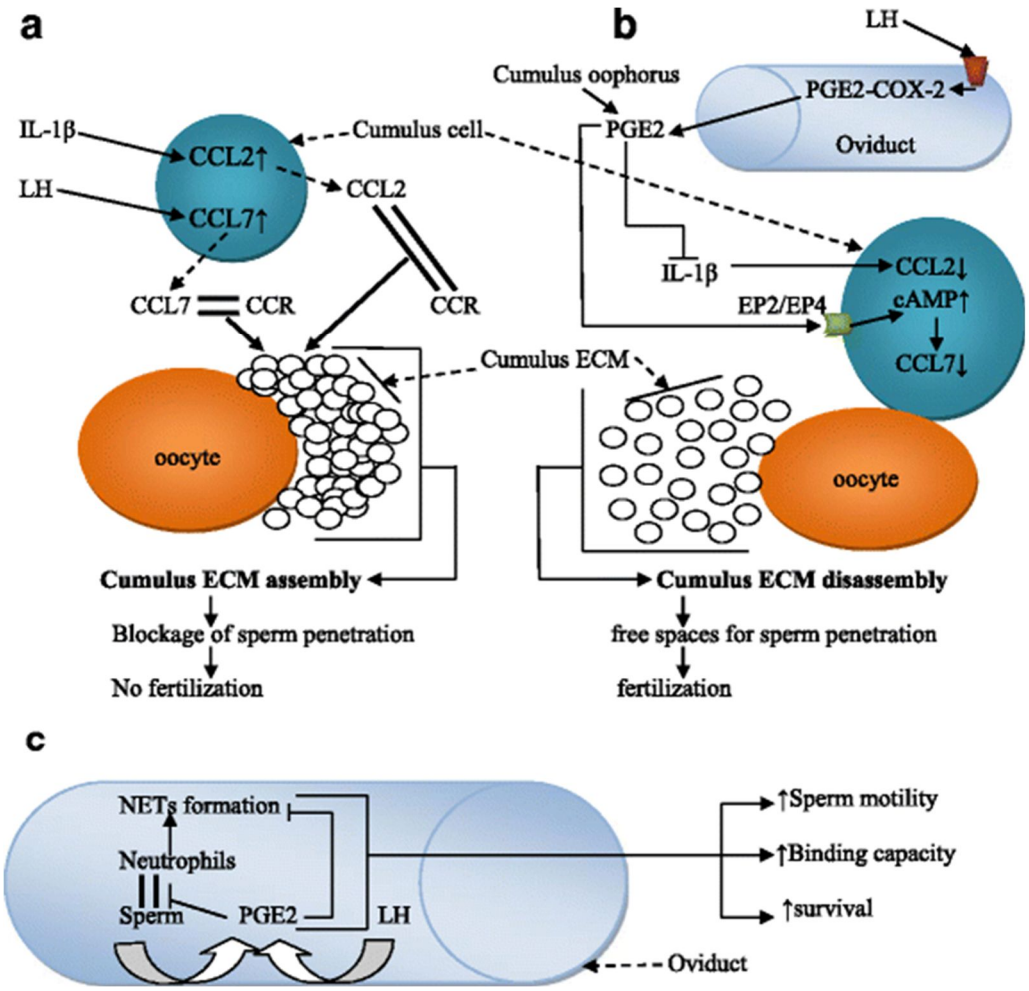

Figure 1.6. Schematic illustration of the effect of PGE2 in the process of fertilization, and the effect of different chemokines secreted from cumulus cells, on sperm activity and oocyte penetration. Note the role of PGE2 in inhibiting interleukin 1B (IL-1B) that reduce chemokine C-C motif ligand (CCL2 and CCL7) that facilitate the disassembly of cumulus cells and fertilization. Source: Niringiyumukiza et al. (2018).

1.8 *In vitro* fertilization

Fertilization is a multiple step process that includes sperm-oocyte penetration, oocyte activation, re-organization of the male chromatin, pronuclei formation and preparation of the one-cell zygote for mitotic division (Lechniak et al., 2008). *In vivo*, sperm is exposed

to different biochemical factors in the female reproductive tract including elevated levels of glycosaminoglycans (GAGs) in oviductal secretions that participate in capacitation (Lenz et al., 1983 and Kim et al., 2013). Also, capacitation of sperm depends on the availability of $HCO3^-$, Ca^+ and soluble adenylyl cyclase (sAC) (Buffone et al., 2014). However, *in vitro*, heparin, BSA and caffeine overcome the capacitation problem for majority of bull sperm (Parish et al., 1988 and Lu and Seidel, 2004). Moreover, addition of creatine to fertilization media enhances the capacitation and fertilizability of mouse spermatozoa through increased tyrosine phosphorylation with elevation of ATP level and sperm motility (Umehara et al., 2018). Furthermore, including antioxidants in fertilization and culture media improves subsequent development of mouse embryos to the blastocyst stage and increases total cell count and inner cell mass in the resultant blastocysts (Truong and Gardner, 2017). In contrast, Goncalves et al., 2010 demonstrated that supplementation of medium with antioxidant impairs pronuclear formation and the development of bovine embryo.

The capacitated sperm adhere to oocytes in the presence of three membrane proteins. Izumo1 on sperm and its receptor Juno on the egg, and Tetraspanin CD9 which accumulates in the egg at the site of sperm adhesion (Sutovsky, 2009; Ghalbi et al., 2014; Kumar et al., 2015 and Antalikova et al., 2015) (**Figure 1.7**).

Ubiquitination of sperm proteins by proteosomes is an important posttranslational modification (PTM) that allows sperm to achieve its goal (Baker, 2016 and Samanta et al., 2016). After adhesion, sperm proteasomes participate in various stages of fertilization such as acrosome reaction, penetration of zona pellucida, mitochondrial sheath degradation and chromatin remodelling (Sutovsky et al., 2004; Rawe et al., 2008 and Kerns et al., 2016). Moreover, in early stages of fertilization the proteasomes translocate to the new pronuclei which participate in chromatin remodelling. However, 10 μM MG132 at the time of insemination inhibits the translocation of proteosomes in treated zygotes resulting in defective pronuclear formation that is mainly due to premature chromosome

condensation (PCC). The latter resulted in defective fertilization in human and bovine zygotes (Rawe et al., 2008).

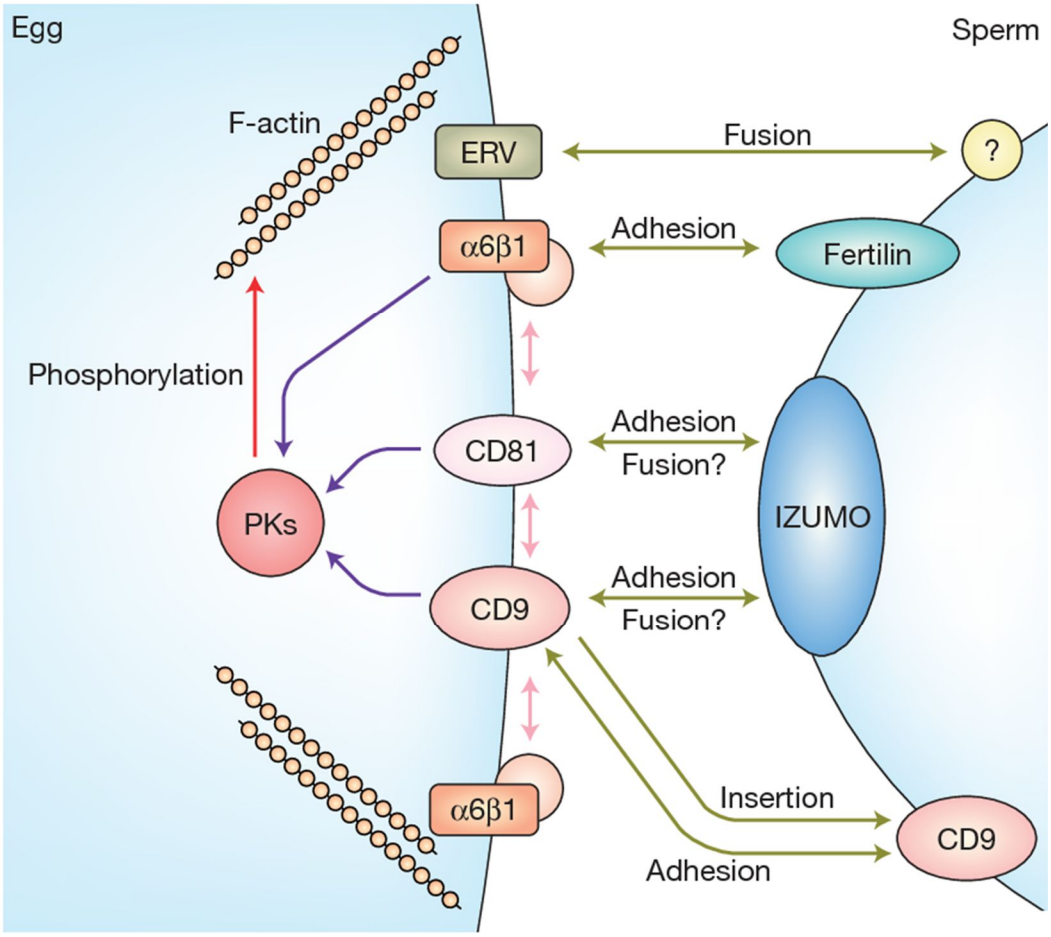

Figure 1.7. Schematic illustration of sperm-oocyte adhesion and fusion and the association of different factors during the fertilization in mammals. Source: Sutovsky (2009).

The time required for sperm penetration of *in vitro* matured bovine oocytes is between 3 to 8 h with an average 6 h from insemination. Also, decondensation of sperm-head takes an additional 1 to 2 h, and pronucleus formation occurs at between 9 to 13 h post insemination, with the first cleavage division at 28 h post insemination (Xu and Greve, 1988; Chian et al., 1999 and Machatkova et al., 2008). Koyama et al. (2014) demonstrated that peak blastocyst yields occurs in cattle when sperm penetration is

achieved at 12.2 h post nuclear maturation. It was demonstrated previously that 18 h or 24 h period of sperm-oocyte incubation is required for *in vitro* fertilization and optimal embryonic development in cattle (Rehman et al., 1994 and Nedambale et al., 2006). However, Ward et al. (2002) found that a 10 h period of gamete incubation is sufficient for optimal fertilization and development of bovine embryos. Also, Kochhar et al. (2003) and Sattar et al. (2011) showed that 6 and 8 h gamete incubation increases the development of zygotes to the blastocysts stage. Moreover, in meta-analytical study of human fertilization, short co-incubation of gametes increased the rate of implantation and pregnancy but had no effect on fertilization (Zhang et al., 2013). In contrast, Long et al. (1994), Krivokharchenko et al. (2001) and Bernald et al. (2011) demonstrated no difference in the developmental capacity of bovine zygotes to blastocysts in a short (1 h, 6 h or 8 h) compared to a conventional (18 h) IVF regime. However, it was found that prolonged gamete incubation is associated with increasing the incidence of polyspermy (Sumantri et al., 1997; Kochhar et al., 2003 and Iwata et al., 2008) and accumulation of deleterious free radicals (Enkhmaa et al., 2009 and Lopes et al., 2010).

1.9 Cell-cycle length and embryonic gene activation

Numerous factors affect *in vitro* post fertilization development of embryos to blastocysts stage. Also, great variability among studies and difficulty to obtain consistent results in embryonic development between laboratories exist (Krisher et al., 1999). While first and second cell-cycle length can determine the ability of zygotes to develop to blastocysts, culture conditions affect cell-cycle length, where suboptimal culture conditions block embryonic development at the 8-16 cell stage in cattle (Holm et al., 1998; Betts and Madan, 2008 and Somfai et al., 2010). The first cell cycle length that end with first cleavage of bovine zygotes determined after extrusion of second polar body post fertilization. Also, the synchronous cleavage post fertilization is controlled by maternal genome that persist till the 4 cell stage. Moreover, embryonic genome activation

commences at the third cell cycle (4-8 cell stage) were protein translation is high during the fourth cell cycle (8-16 cell stage) (Barnes and Eyestone, 1990 and Meirelles et al., 2004). Holm et al. (1998) demonstrated that duration of the first four cycles in viable bovine embryos is 32.0±3.9, 8.8±1.6, 10.8±4.7 and 47.7±11.8 h respectively, with no difference in cleavage intervals between male and female embryos. Also, a high proportion of 3-4 cell stage embryos is achieved by 44 h post insemination in cattle (Carrocera et al., 2016). The length of the fourth cell cycle, when maternal to zygotic transition (MZT) occurs, is shortened with the reduction of oxygen from 20% to 5% in bovine embryos cultured *in vitro* (40-50 h to 9 h respectively). Also, reduction of oxygen tension from 20% to 5% improved the development of bovine embryos to the blastocyst stage when embryos were beyond the 8-cell stage. Whereas more embryos arrested in the case of high oxygen tension in cattle (Lequarre et al., 2003), and human (Kirkegaard et al., 2013). Moreover, proteasome activity is important during MZT and application of MG132 for the first 9 h post insemination delays the onset of zygotic transcription and reduces development of mouse embryos (Shin et al., 2010 and Higuchi et al., 2018). Furthermore, the latter study showed that the activity of proteasomes in treated embryos recovers gradually between 12-24 h post insemination.

Recently there has been great interest in conducting RNA-Seq analysis to determine the proportion of transcripts that are of maternal or embryonic origin during the preimplantation period as an indicator of maternal to embryonic transition (MET) in bovine (Graf et al., 2014a, b and Lavagi et al., 2018) (**Figure 1.8**).

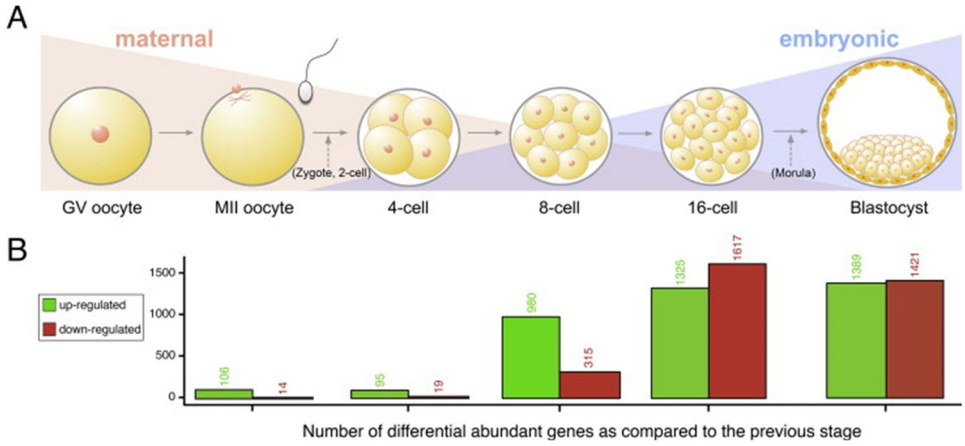

Figure 1.8. Schematic diagram of differential gene expression during preimplantation development as a tool for the determination of maternal to embryonic transition (MET) of bovine embryos cultured *in vitro* as determined by RNA-Seq analysis. Source: Graf et al. (2014).

The lag phase in which longer Gap2 phase is present is related to embryonic gene activation (EGA) that occurs during the fourth or fifth cell cycle. This might be related to cell allocation or the differentiation of embryonic cells into inner cell mass (ICM) and trophectoderm (TE) and apoptosis in bovine blastocysts (Sugimura et al. 2012 and Yao et al., 2018).

1.10 Embryonic cell lineages and pluripotency

Divergence of the two-cell lineages into trophectoderm (TE) and inner cell mass (ICM) begins when cellular polarity occurs at around the 9-15 cell stage in the bovine embryo (Nicholas and Smith, 2012). The surface microvillous polarization is an early indicator of differentiation between the two cell lineages in cattle (Koyama et al., 1994) and in mouse (Humięcka et al., 2017). Following compaction, the trophoblast transfers fluid into the embryo forming a blastocoel and, after cavitation, the ICM is partitioned into the epiblast and hypoblast, or primitive endoderm (PrE) (Nicholas and Smith, 2012). In late blastocysts, the epiblast cells are pluripotent and contribute to the three germ layers which have the ability to convert to any cell type of the mature body (Condic, L. 2014). Different genes participate in maintaining pluripotency of the ICM. Among those

important genes that protect or maintain the pluripotent state of ICM is Sex determining region Y-box 2 (*SOX2*) (Zhang and Cui, 2014). Deficiency of this transcript leads to embryonic lethality in the mouse (Avilion et al., 2003). Also, analysis of gene expression profiles in the two-cell lineages indicates that, in keeping with the mouse, *SOX2* is a predominant transcript in the inner cell mass of bovine embryos (Khan et al., 2012 and Nagatomo et al., 2013). Moreover, *SOX2 i*s an early marker for cell lineages in mouse (Guo et al., 2010) and bovine embryos (Yuan, Y. 2018). Furthermore, Rizzino and Wuebben, 2016 reported that expression of *SOX2* (mRNA and protein) in zygotes begins at morulation, and both *SOX2* and *Oct4* are required during cell lineage decision of mammalian embryogenesis (**Figure 1.9**).

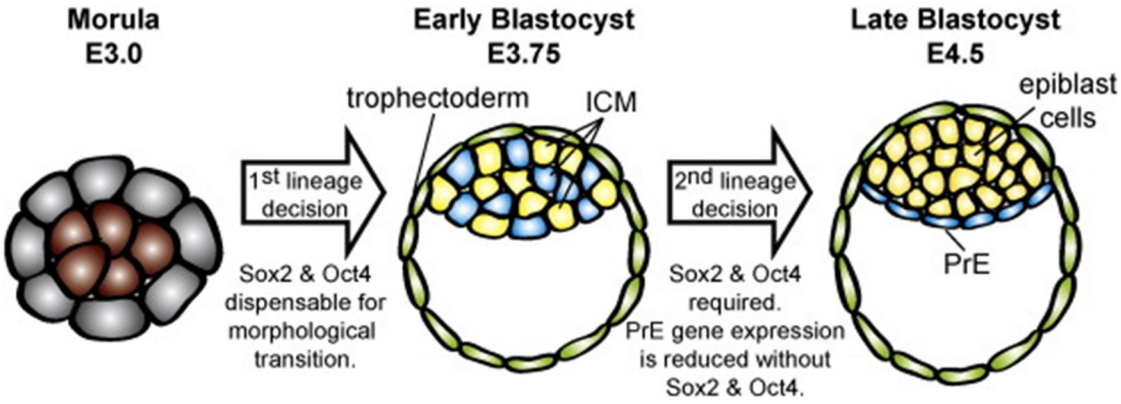

Figure 1.9. Schematic diagram of the role of *Sox2* and *Oct4* in the cell lineage decision during mammalian embryogenesis. Source: Rizzino and Wuebben, (2016).

There are numerous factors that affect inner cell mass and total cell number of embryos cultured *in vitro*, which include the presence of growth factors or cytokines. Epidermal growth factor (EGF) and Insulin-like growth factor (IGF-I) are among the factors that enhance proliferation and increases inner cell mass of bovine embryos cultured *in vitro* (Moreira et al., 2002; Sirisathien et al., 2003 and Sakagami et al. 2012). However, other studies reported no difference in cell number after treatment with either EGF or IGF-I during culture (Ahumada et al., 2013 and Arat et al., 2016).

Administration of Colony stimulating factor (CSF2) from D5 of IVC alters the expression of different genes related to pluripotency and differentiation and increases inner cell mass of bovine embryos cultured *in vitro* (Loureiro et al., 2009, 2011 and Hansen et al., 2014a). However, Dobbs et al. (2013a) found no effect of CSF2 on the expression of *NANOG* and *SOX2* in bovine embryos but speculate that other pluripotency genes could be altered following administration of CSF2.

1.11 Embryo quality and assessment

While the early cleavage stages of embryo development during in vitro culture can provide a morphological indicator of quality (Lechniak et al., 2008) (**Figure 1.10**), subsequent development *in vitro* might show different morphological merit that determine the quality of the resultant embryos.

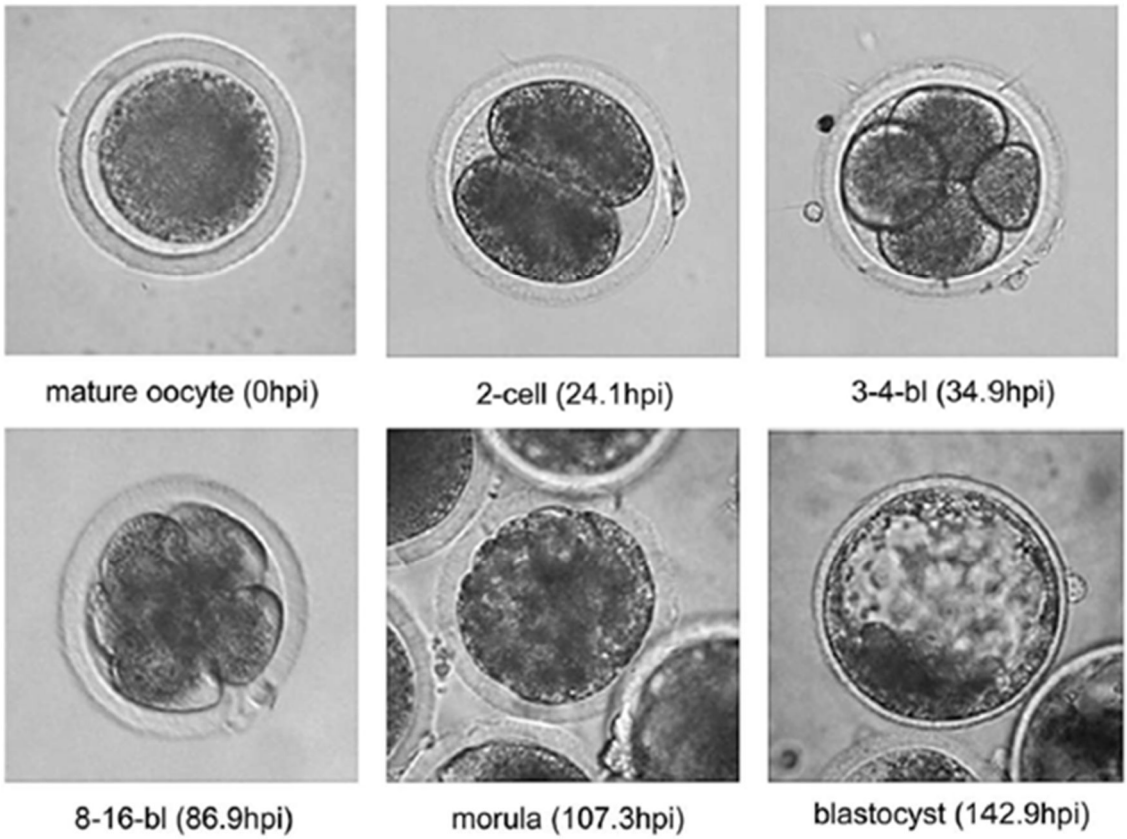

Figure 1.10. Mean timing of embryonic stage in cattle during preimplantation period *in vitro*. (hpi= hours post insemination). Source: Lechniak et al. (2008).

Embryo quality is determined in humans during the early preimplantation period and can be defined by the number of blastomeres, blastomere fragmentation and blastomere symmetry (Antczak and Van Blerkom, 1999; Sela et al., 2012 and Machtinger et al., 2015). Different approaches are utilized for the assessment of bovine embryo quality during preimplantation including, morphological (Lindner and Wright, 1983), scanning electron microscopy (Shiku et al., 2001), micro RNA profile (Kropp et al., 2014), cell division timing (Farin et al., 1995), embryo metabolism (Thompson et al., 2016) and transmission electron microscopy (López-Damián et al., 2008). However, to date there is no definitive, objective, non-invasive and practical technique to evaluate embryo quality in humans and animals (Rocha et al., 2016). Also, different methods of classification and differences in experience of the examiners result in various assessment outcomes (Gardner and Schoolcraft, 1999; Hasler, 2000; Kovacic and Vlaisavljevic, 2012; Bo and Mapletoft, 2013 and Rocha et al., 2016).

Morphological classification according to the stage and age of embryos with differential staining of blastocysts and counting of ICM and TE cells is a very useful method for the assessment of the quality of blastocysts produced *in vitro*. The total cell count in early, expanded and hatched blastocysts were 44 ± 18, 77 ± 33 and 121 ± 46 cells respectively (Maylem et al., 2017). Whereas, Iwasaki et al. (1990) reported that inner cell mass in those blastocysts was 8 ± 5, 11 ± 7 and 16 ± 8 cells respectively. However, utilizing different culture systems, Thouas et al. (2001) reported that total cell number in D7 expanded and D8 Hatched blastocysts were 151.3 ± 5.48 and 217.8 ± 8.75 cells respectively. Corresponding number of cells within the inner cell mass was 34.2 ± 1.84 and 49.9 ± 2.83 respectively with ICM: TE ratio of around 1:3.

1.12 Embryokines and embryonic development

Embryokines are defined as molecules produced by the reproductive tract that include hormones, growth factors and cytokines which regulate embryonic development and growth (Hansen et al., 2014, Tribulo et al., 2018). Embryokines that have an vital role during pre and post implantation development of bovine embryos include IGF (Bonilla et al.,2011 and Velazquez et al., 2009, 2012); Dickkopf-1 (DKK1) (Denicol et al., 2014 and Hansen et al., 2014); Colony-Stimulating Factor 2 (CSF2) (dMoras and Hanssen, 1997; Loureiro et al., 2009; Neira et al., 2010; Dobbs et al., 2013 and Sequeira et al., 2017) (**Figure 1.11**) and Transforming Growth Factor B1 (TGFB1) (Neira et al., 2010 and Moreno et al. 2015).

In addition to their role in the regulation of fertilization, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF or CSF2) and Transforming Growth Factor B1 (TGFB1) play important roles in the development of embryos during the pre- and post-implantation periods (Neira et al., 2010; Sequeira et al., 2017 and Samanta et al., 2018).

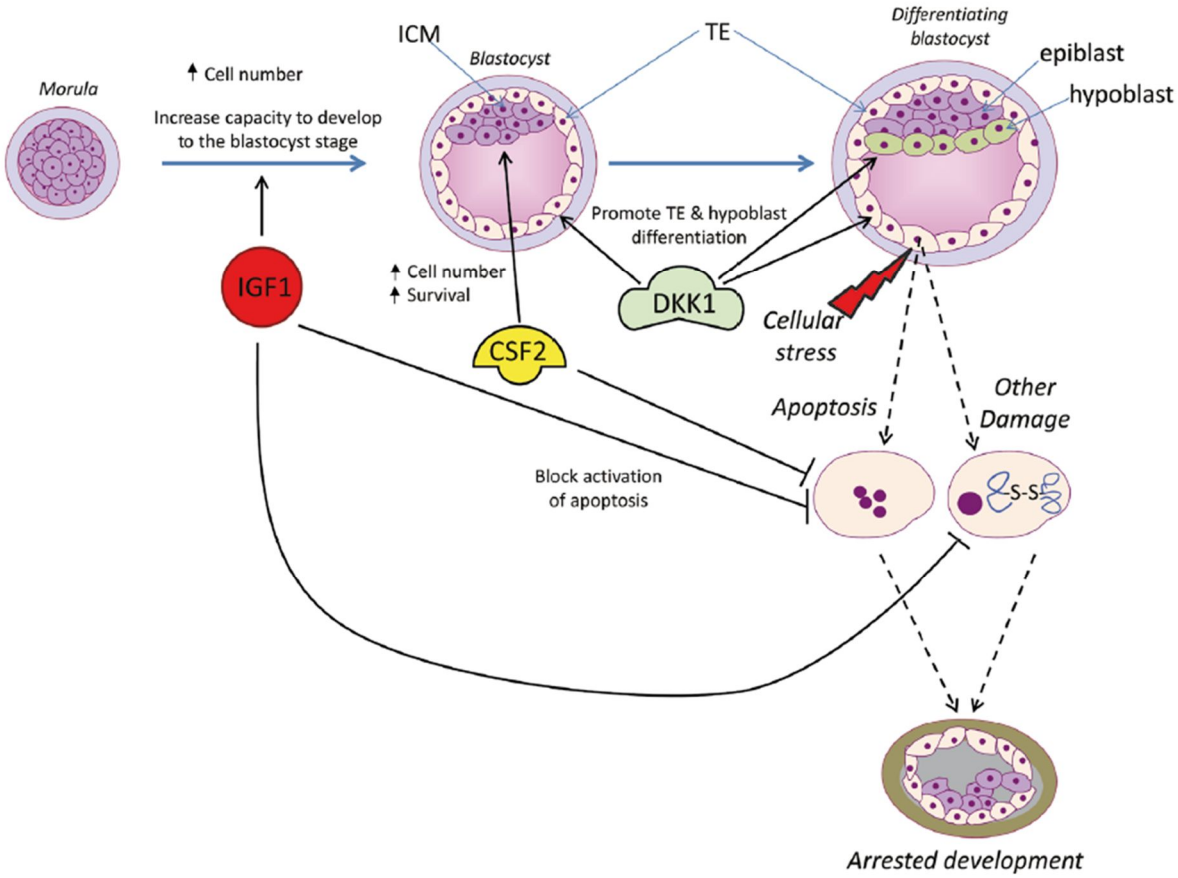

Figure 1.11. Schematic illustration of some events in bovine embryonic development beyond morulation and the role of Insulin Growth Factor (IGF), Colony Stimulating Factor (CSF2) and Dickkopf -1 (DKK1). Note that CSF2 inhibit the apoptosis of ICM and increase their cell number and survival of embryo. Source: Hansen et al. (2014).

CSF2 is a monomeric glycoprotein that contains 127 amino-acid residues with two glycosylation sites, and has a molecular weight around 14 kDa depending on the degree of glycosylation. Also, CSF2 is produced by many cell types such as macrophages, granulocytes, endothelial cells, fibroblasts, smooth muscle, T-Lymphocytes and mast cells. Moreover, CSF2 mediates its effects by binding to heterodimeric receptors that contain α and β c-subunits shared with interleukins (IL-3 and IL-5) (Miyajima et al., 1993 and Sherbet, 2011). Binding CSF2 to their receptors resulted in stimulation of multiple pathways involved in cell-cycle progression including Ras/Raf/MAPK, PI-3 Kinase, JAK/STAT, JNK/SAPK, and p38 signalling pathways (de Groot et al., 1998; Rumore-Maton et al., 2008 and Jeong et al., 2014).

Cytokines such as IL-3 and CSF2 promote the synthesis of GLUT1 (membrane Glucose transporter) in haematopoietic cells (Zambrano et al., 2010 and Sarrazy et al., 2016), and increase glucose uptake in Xenopus oocyte (Dhar-Mascareño et al., 2003). Also, the reduction in expression of GLUT1 can trigger the apoptosis in murine blastocysts (Chi et al., 2000). CSF2 is expressed in the uterine epithelium and remains high during pre and post implantation period in human (Chegini et al., 1999 and 1999a and Hoff et al., 2016), cattle (Emond et al., 2004; de Moraes et al., 1999 and Tribulo et al., 2018) and mice (Robertson et al., 1996 and Moldenhauer et al., 2010). Also, CSF2 increases the level of maternal pregnancy recognition factor (interferon-tau) that maintains pregnancy in cattle (Michael et al., 2006) and sheep (Imakawa et al., 1993). Moreover, CSF2 increases endometrial thickness and hence implantation success in women (Gleicher et al., 2011; Tehraninejad et al., 2015 and Sarvi et al., 2017).

Although some studies reported that CSF2 has no effect on the development of embryo during preimplantation (Robertson et al., 2001; Rooke et al., 2005; Desai et al., 2007; Hickman et al., 2011 and Elaimi et al., 2012), its positive role has been shown in different mammalian species including humans (Sjoblom et al., 1999 and Economou et al., 2017), cattle (dMoras and Hanssen, 1997; Loureiro et al., 2009; Neira et al., 2010 and Sequeira et al., 2017), Yak (Wen et al., 2017), sheep (McGuire et al., 2002), pig (Kwak et al., 2012a, b; Lee et al., 2013 and Cai et al., 2015) and mouse (Robertson S. A., 2007, and Chin et al., 2009). However, Dobbs et al. (2013) demonstrated that CSF2 has a positive effect on blastocyst yield in bovine only when the level of development is low. The mechanism by which CSF2 increases the number of bovine zygotes reaching the blastocyst stage is believed to be associated with upregulation of anti-apoptotic genes (*PRKAR2B, NT5E* and *PGR*), and downregulation of proapoptotic genes (*MADD, RIPK3, NOD2* and *CREM*), cell cycle regulation and differentiation (Loureiro et al., 2011 and

Hansen et al., 2014), and upregulation of anti-stressor genes during preimplantation in mouse and cattle (Chin et al., 2009 and Wen et al., 2017).

On the other hand, TGFB1 is a member of TGFB family which includes nodal, activin, inhibin, Growth Differentiation Factors (GDFs) and Bone Morphogenic Proteins (BMPs). TGFB1 is one of several isoforms (TGFB1, TGFB2 and TGFB3) which play an important role in development, immune response, wound healing and inhibition of tumour cell proliferation (Shi et al., 2011 and Finnson et al., 2013). TGFB is secreted as a mature homodimer combined with two components. The first component is a propeptide called latency associated protein (LAP) that inhibits the high affinity of TGFB for binding to its membrane receptors. The second component is latent TGFB binding protein (LTBP), or fibrillin, that binds to LAP in disulfide bond which is required for effectiveness of latent TGFB (Chen et al., 2005). Elevation of LTBP and LAP increase *SMAD2/3* expression in the uterus and maintains implantation sites in mouse embryos (Maurya et al., 2013 and Rodriguez et al., 2016). Moreover, TGFB family members act as paracrine and autocrine factors during the pre- and post-implantation periods (Jones et al., 2006) (**Figure 1.12**).

The ligands of the TGFB superfamily bind to the receptor complexes composed of TGFB type I (RI) and type II receptors (RII), also called activin-like kinase (Alk). Seven type I, Alk 1–7, and five type II receptors have been recognised in mammals. The binding of TGFB activates serine/threonine kinase of RII domain, that phosphorylates RI on specific serine and threonine residues in the juxta membrane serine and glycine-rich Gs domain. Activation of Smads phosphorylation resulted in their nuclear translocation. TGFB1 uses TGFB1 receptors (Alk5, T*β*RII) and phosphorylation of *SMAD2*/*3* occurs post activation of these receptors. Then after, *SMAD4* forms heteromeric complexes with *SMAD2/3* that activates transcription and expression of target genes (Massague et al., 2005 and Saha et al. 2008).

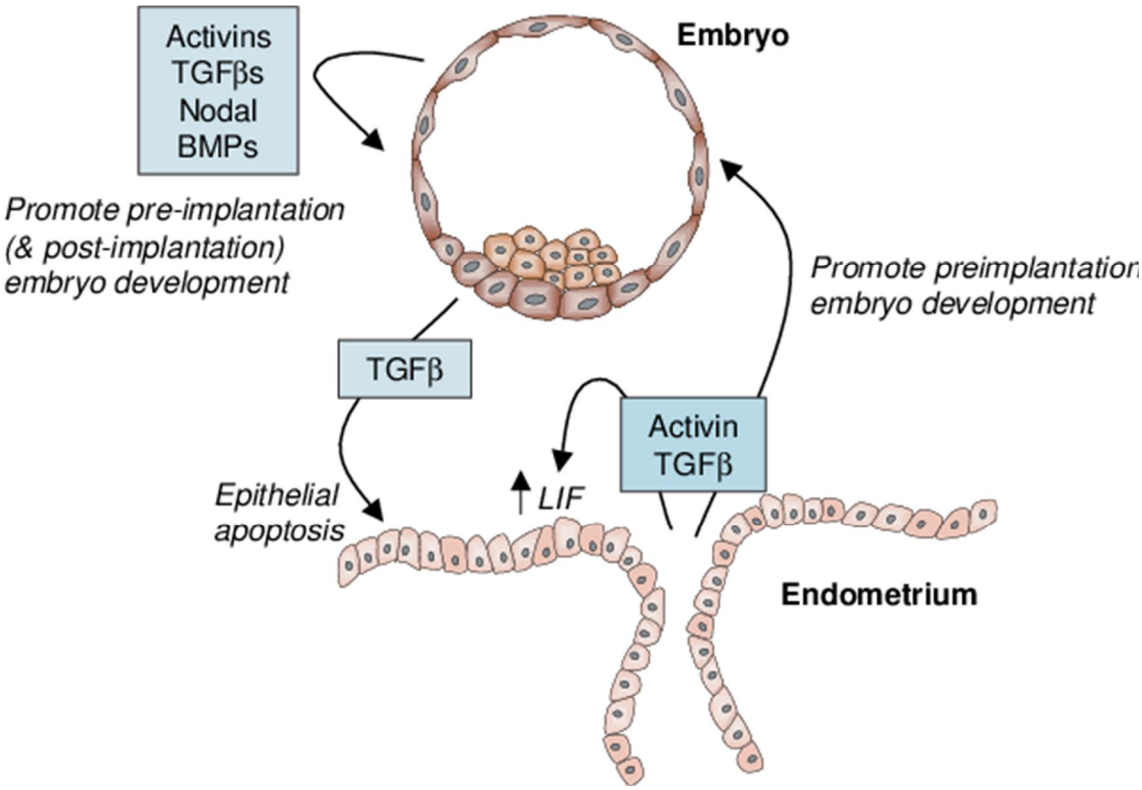

Figure 1.12. Illustrative diagram of proposed paracrine and autocrine effects of TGFB family members during pre- and post-implantation period and their effect on enhancing the production of pro-implantation cytokine Leukemia inhibitory factor (LIF) from the epithelial cell lining of the endometrium. Source: Jones et al. (2006).

Kim et al. (2005) reported that activation of *SMAD3* in response to TGFB on cytostatic signalling in cell lines represses growth promotion transcription factors and activates cell cycle inhibitors. Also, the latter showed that *SMAD3*:*SMAD2* ratio plays a crucial role in TGFB activity. Elston and Inman, (2012) summarised canonical and non-canonical signalling pathways of TGFB1 (**Figure 1.13**).

The pleiotropic effects of TGFB ligands seem to be a result of distinctive canonical and non-canonical signalling pathways. Canonical *SMAD* pathways inhibit tumour progression, cell proliferation and promotes apoptosis. However, opposite action occurs when non-*SMAD* pathways are activated (Sherbet, 2011).

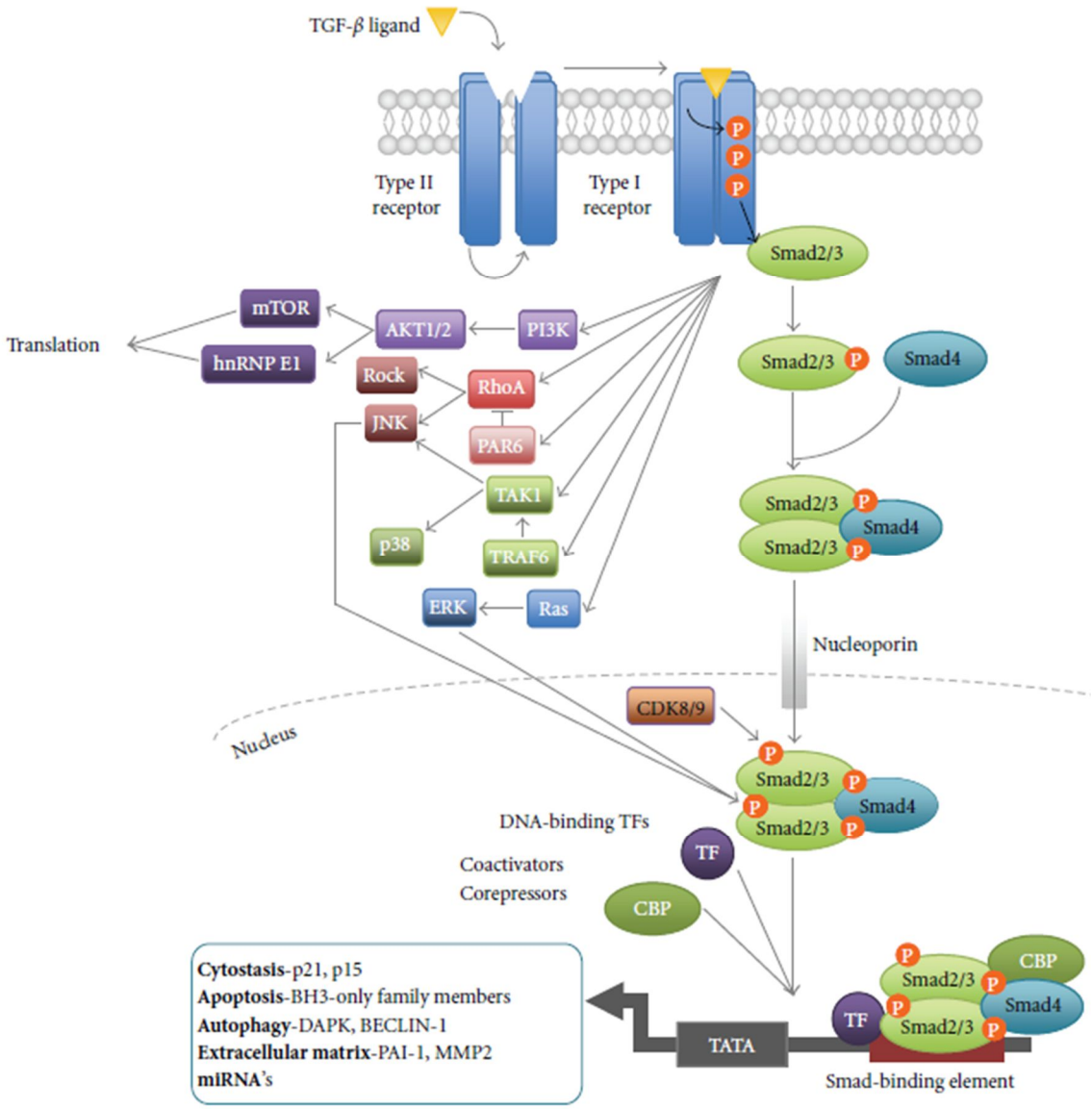

Figure 1.13. Schematic diagram of canonical and non-canonical signaling pathways of TGFB1. Source: Elston and Inman, (2012).

Administration of 50 ng ml^{-1} TGFB1 at D4 of *in vitro* culture has a beneficial effect on the development of bovine embryos to the blastocysts stage (Neira et al., 2010 and Moreno et al., 2015). However, Keefer (1992) found that TGFB1 at 2 ng ml^{-1} had no effect on the development of bovine embryos or total cell number of the resultant blastocysts. Also, it has been found that early treatment of bovine embryos in the first 48 h of culture with 50 or 100 ng ml^{-1} TGFB1 had no effect on the development of zygotes to the blastocyst stage (Barrera et al., 2018).

1.13 Primary Sex ratio

Primary sex ratio is defined as the ratio of male to female embryos that are determined when X or Y chromosome bearing sperm fertilise the oocyte (Iwata, H. 2012). *In vivo*, Grant et al. (2008) reported that the sex of embryos is influenced by the mother in cattle, where high testosterone levels in follicular fluid preferentially selects Y chromosome-bearing spermatozoa. In contrast, *in vitro* fertilization environment leads to erroneous epigenetic modification through impairment of imprinted X chromosome inactivation (iXCI) by reduction of Ring finger protein 12 (Rnf12) / X-inactive specific transcript (Xist) that skews the sex ratio in preference of male embryos in mouse (Tan et al., 2016). Among the numerous factors that might affect sex ratio during *in vitro* fertilization of bovine embryos the sire, *in vitro* maturation status of the oocyte, incubation period of gametes during fertilization and culture media are discussed herein.

Alomar et al. (2008) and Rorie et al. (2014) demonstrated a sex ratio variation between bull semen. Also, differences in sex ratio between bull semen batches from the same bull has been reported (Chandler et al., 1998, 2002). However, Madrid-Bury et al. (2003) and Sattar et al. (2011) showed no differences between bulls and ejaculates in the sex ratio of resultant bovine embryos cultured *in vitro*. On the other hand, Dominko and First, (1997, 1997a) and Gutierrez-Adan et al. (1999) reported that fertilization of bovine oocytes immediately post polar body extrusion increases the percentage of female embryos, and the delay of *in vitro* maturation for an extra 8 h skews the ratio towards male embryos. Also, extension of maturation period to 34 h, instead of 16 or 22 h, increases male ratio in the resultant bovine embryos that are cultured *in vitro* (Agung et al. 2006). In terms of incubation period of gametes for fertilization, Kochhar et al. (2003) demonstrated that short incubation periods for 6 h increases the developmental capacity of bovine embryos and elevates male ratio in the resultant blastocysts. However, Sattar et al., (2011) reported that the sex ratio in relation to different incubation times is not

different, but more female embryos were present when development level was low in culture.

The exposure of sperm to cumulus cells pre denudation of oocyte or during *in vitro* fertilization of complete Cumulus Oocyte Complexes (COCs) shifts the primary sex ratio into male in cattle is attributed in part to differences in capacitation status of X and Y bearing sperm (Iwata et al., 2008). Culture media composition also affects sex ratio of embryos. Serum in culture media enhances the development of bovine embryos to the blastocyst stage and increases overall male survival (Gutierrez-Adan et al., 2001a). Also, glucose supplementation during bovine embryo culture shift the sex ratio into male with a significant loss of female embryos (Gutierrez-Adan et al., 2001 and Kimura et al., 2005). Moreover, it has been found that glucosamine skewed the sex ratio towards males when added post maternal zygotic transition in cultured bovine embryos (Kimura et al., 2008). Furthermore, addition of CSF2 to culture media from D5 of culture enhances the development of female embryos in cattle (Siqueira and Hansen, 2016). In humans, the developmental advantage of male over female embryos in IVF, sperm preparation technique and molecular composition of zona pellucida- that expose oocyte to be more susceptible to fertilization with Y-bearing sperm- are among the factors that participate in elevation of sex ratio (Tarin et al., 2014).

2. Hypothesis

With the foregoing discussion in mind, the current thesis advances the hypothesis that:

a. Increasing mitochondrial DNA replication and reducing proteasomal degradation during *in vitro* maturation produces functionally active mitochondria that enhances the developmental competence of oocytes and improves post fertilization development of bovine embryos.
b. Enhancing the developmental competence of D5 embryos by utilizing embryokines will promote blastocyst yields and improve embryo quality.
c. Combining these approaches and increasing the developmental competence of oocytes and embryos during culture will result in high yields of female blastocysts.

These hypotheses were tested by addressing the following objectives:

3. Objectives

a. To investigate the effects of SNP and MG132 separately and in combination on ATP content, first polar body extrusion, mtDNA copy number and post-fertilization development of bovine embryos cultured *in vitro*.
b. To assess the effects of combinations of TGFB1 and CSF2 on the development of D5 embryos.
c. To determine the best combination of approaches from above on embryo development and primary sex ratio at D8 of bovine *in vitro* culture.

Chapter 2: Effect of sodium nitroprusside and MG132 on oocyte maturation and development of bovine embryos

2.1 Introduction

Producing functionally active mitochondria by lowering their degradation and increasing mtDNA copy number might increase the developmental competence of bovine oocytes during *in vitro* maturation. ATP production and mtDNA replication is critical for the determination of developmental competence of porcine oocytes (Lee et al., 2014a). mtDNA replication occurs during oocyte growth and maturation prior to metaphase II and in trophectoderm cells around the blastocyst stage. Nitric oxide (NO) is known to stimulate the PGC1–NRF1–TFAM (peroxisome proliferative activated receptor, gamma, coactivator 1 – Nuclear respiratory factor 1- Transfer factor A, mitochondrial) pathway. Binding of PGC1 coactivator and NRF1 increases mitochondrial TFAM and initiates mtDNA transcription (Scarpulla, 2008 and St John et al., 2014). Few studies have investigated the effect of NO donation on mtDNA copy number. However, Nisoli et al. (2004) and Tengan et al. (2012) found that stimulation of mitochondrial biogenesis through NO pathway produces functionally active mitochondria in rat myocytes. Also, NO deficiency during maturation leads to apoptosis of bovine oocytes cultured *in vitro* (Schwarz et al., 2010).

Bilodeau-Goeseels, (2007) reported that the NO donor, Sodium Nitro-Prusside (SNP), at low concentrations (0.01 µM), increases the percentage of oocytes that resume meiosis, but in another study it delays GVBD in bovine oocytes post 9 h period of maturation (Schwarz et al., 2014). On the other hand, Viana et al. (2007) showed that SNP at 10 µM during oocyte maturation had a beneficial effect on the development of bovine embryos. In addition, the latter study demonstrated that SNP did not affect nuclear maturation but led to a significant increase in blastocyst yield per oocyte (40% vs 27% for the control group). From these studies we can conclude that utilizing SNP at

10 µM might increase functionally active mitochondria that have high mtDNA copy number and lead to developmentally more competent oocytes.

You et al. (2012a) reported that, late in maturation, proteasomes contribute in the reduction of functional properties of bovine oocytes by inducing an 'aging-like' effect and that the use of MG132 (a proteasome inhibitor) reduces such effects. In addition, treatment with MG132 increases abundance of Mitofusins (Mfn-1, 2), which are GTPases embedded proteins in mitochondrial membranes, and the rate of mitochondrial fusion that can compensate for damage and reduce mitophagy (Tanaka et al., 2010). Furthermore, mitochondrial fusion is an essential process for the development of mouse embryos (Chen et al., 2003). MG132 upregulates and downregulates different proteins during *in vitro* maturation of bovine oocytes. GAPDH, which is involved in glycolysis, is among the upregulated proteins as is P4HB which has an anti-apoptotic effect. On other hand, late during *in vitro* maturation, MG132 downregulates other proteins including CDK5 which, if activated, can led to apoptosis (You et al., 2012a). However, Zhou-Stache et al. (2002) observed that MG132 at a concentration of 5 µg ml^{-1} (~10 µM) for 30min prevented the degradation of IKB, an inhibitor of nuclear factor Kappa-B cell ($NF\text{-}_kB$) in endothelial cells of the human umbilical vein. This prevented the activation of $NF\text{-}_kB$ and so mediated cell protection and survival.

Administration of 10 µM MG132 during late oocyte maturation increases the developmental competence of oocytes and has no negative effect on embryo viability in the cow (You et al., 2012a), Yak (Xiao et al, 2014), porcine (You et al., 2012 and Shen et al., 2017) and rat (Ono et al., 2011). Few studies have considered the effects of MG132 on mtDNA copy number in cattle. However, Kansaku et al. (2017) reported that treatment of oocytes of young but not old cows with 10 µM MG132 increases mtDNA copy number. Based on these studies it might be concluded that treatment of bovine oocytes late in maturation with 10 µM MG132 would have a beneficial effect on oocytes through upregulating cell survival proteins. Also, it might participate in the reduction of

mitochondrial fusion and hence maintain adequate DNA copy number that ensures the production of required ATP for the development of embryos. The aim of the current study, therefore, was to investigate the effect of a novel combination of SNP and MG132 on mitochondrial activity that might increase oocyte competence and improve development of bovine embryos cultured *in vitro*.

2.2 Material and methods

2.2.1 Chemicals

All chemicals used in this study were purchased from Sigma Aldrich chemical Co. (Darmstadt, Germany) unless otherwise stated. The ATP kit (FLASC -ATP bioluminescence somatic cell assay kit, Sigma) was accompanied with ATP standard (FLASS), dilution buffer (FLAAB), assay mix (FLAAM), and releasing reagent (FLSAR) that was prepared according to the manufacturer instructions. The main reagents used in this study is sodium nitroferricyanide dihydrate (SNP-228710), MG132 (*N*-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal - C2211) and Dimethyl sulfoxide (DMSO-D2438).

2.2.2 COCs collection and *in vitro* maturation

Bovine ovaries were received from a local slaughterhouse (Pickstock Telford Ltd., Telford, UK) in warm PBS and transferred to the lab within 2-3 h. Follicles 4-9 mm in diameter (**Figure 2.1A, B**) were aspirated using a 19-gauge needle attached to a 10 ml syringe. Recovered COCs were selected morphologically based on the criteria of Hazeleger et al. (1995) (see Appendix II-A), and washed with HEPES buffered (M-199) medium (GIBCO, BRL, Grand, Island, NY) supplemented with 10% heat inactivated fetal calf serum (FCS) and gentamycin (50 mg ml^{-1}). COCs were matured in Nunc 4 well dishes (Thermo Fisher Scientific, UK) with 500 µl per well of bicarbonate buffered medium (M199, GIBCO, BRL, Grand, Island, NY) plus 10% heat inactivated FCS,

Gentamycin (50 mg ml^{-1}), Epidermal growth factor(EGF) (10 µg ml^{-1}), Sodium pyruvate (100 mg ml^{-1}), Cysteamine (1.1 mg ml^{-1}), Pluset (FSH & LH; Calier, Spain) (5 IU ml^{-1}) and Glutamax (200 mM-Invitrogen), and incubated in a humidified incubator at 38.5 ºC, in 5% CO_2 in air for 22-24 h. (**Figure 2.1C**).

2.2.3 First Polar body extrusion

Expanded matured oocytes (**Figure 2.1D**) were fully denuded from cumulus cells using 0.3% hyaluronidase and then vortexed for 3min, washed in HEPES-SOF and transferred to Phosphate Buffer Saline-Polyvinylpyrrolidone (PBS-PVP 0.1% w/v). First polar body (PBI) extrusion was assessed utilizing a stereomicroscope (Leica MZ12.5, Germany) and magnified images were captured using contrast inverted microscope (**Figure 2.1E, F**).

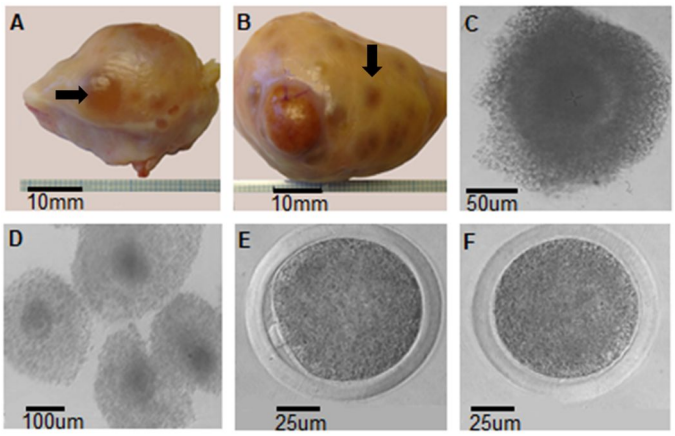

Figure 2.1. Ovaries from abattoir with single 9 mm follicle (A) and multiple 4mm follicles (B), Graded COCs prior maturation (C), Expanded matured COCs (D), extruded (E) and non-extruded first polar body in fully denuded oocyte (F).

2.2.4 ATP content

ATP content of completely denuded single oocytes was measured using a bioluminescence somatic cell assay kit (Sigma, Darmstadt, Germany) according to the manufacturer instructions and as previously described (Nagano et al., 2006). Briefly, fully

denuded oocytes were transferred individually into 0.2 ml microcentrifuge tubes containing 25 µl of PBS-PVP 0.1%, heated to 95ºC for 3min and stored at -80°C until assayed. On the day of assay, ATP stock (Firefly Luciferase ATP Assay Standard-FL-AAS) (1 mg ml^{-1}) was diluted with PBS-PVP to prepare duplicate eight-point standards (0-20 pmol) 25 µl each and heated to 95ºC for 3min (**Figure 2.2**). Samples, standard, releasing agent (FLSAR) and diluted assay mix (FLAAM) were mixed with buffer (FLAAB) then transferred to ice. Samples and standards were then uploaded to a 96 well plate and 50 µl of releasing reagent added to each and left for 5 min on ice to react. Using a multichannel pipette, 100 µl of diluted assay mix were added to each reaction well and allowed to stand for 5min at room temperature for reaction before loading in the luminometer. ATP content was quantified by measuring luminescence (Berthold LB 96v luminometer) produced in an ATP-dependent luciferin-luciferase assay. Data were elicited by a standard curve generated in Excel software for each plate.

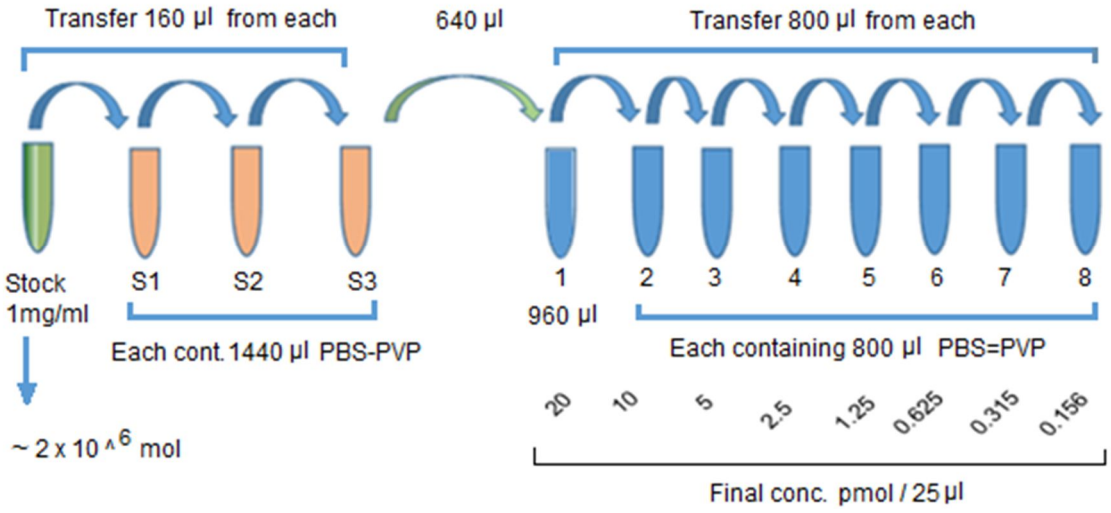

Figure 2.2. Illustrative diagram for the preparation of serial dilution of standards for measurement of ATP content in single bovine oocytes utilizing bioluminescence somatic cell assay.

2.2.5 Quantitative Polymerase Chain Reaction (qPCR)

Absolute quantification of mitochondrial DNA was performed by qPCR for control and treated single oocytes. Sequences of 16S mitochondrial encoded ribosomal RNA (mt-rRNA) of the *Bos taurus* mitochondrial genome were selected from GenBank (https://www.ncbi.nlm.nih.gov, Accession number AY526085) as previously described (Chiaratti et al., 2010). Primers were designed using Primer Express software version 3.0.1. Selected forward and reverse primers A and B (**Table 2.1**) were supplied by Eurofins Genomics (GmbH, Anzinger Str. 7A, 85560, Ebersberg, Germany). Primers were tested electrophoretically before used for molecular analysis.

Table 2.1. Forward and reverse primers (A and B) utilized in standard preparation.

Primers	Sequences 5' - 3'	Position
A	Forward: CGGTATCCTGACCGTGCAAA	2367
	Reverse: TATTCTCCGAGGTCACCCCA	2609
B	Forward: TGACCGTGCAAAGGTAGCA	2375
	Reverse: TATTTGTGCATTCCCGCCTC	2506

Plasmids with inserted fragments were utilized for real time PCR as previously described by Chiaratti et al. (2010) with modifications. Briefly, 5 matured denuded oocytes were lysed in 6 µl lysis buffer (10 mM Tris-HCl with pH8, 1% Triton X-100, and 0.016 U proteinase K) and incubated at 55ºC for 30min followed by 95ºC for 5min. A 243 bp PCR fragment was generated by performing PCR in 25 µl of reaction mixture containing 0.5 µM of each forward and reverse primers A, GoTaq master mix (Promega), and DNA from lysed oocytes. PCR was performed with initial 95ºC for 2 min followed by 38 cycles of 94ºC for 15 sec, 58ºC for 15 sec, and 72ºC for 30 sec with final step of 72ºC for 10 min. PCR products were run on a gel to confirm the size and gel purified using MinElute Gel Extraction kit (Qiagen) according to the manufacturer's instructions. Purified PCR

products were ligated into pGEM-T Easy vector (Promega) according to the manufacturer's instruction. Then the vector was transformed into JM109 High efficiency competent cells (Promega) and cells then plated onto Lysogeny broth or LB agar with ampicillin/IPTG/X-gel (Fisher) for blue/white colonies screening to identify the transformants that contained recombinant plasmid. The colonies were then tested for mitochondrial DNA insertion by PCR in a total reaction of 10 µl containing Immomix Red (Bioline), 0.5 µM of each forward and reverse primers A. PCR conditions were 95^{O}C for 10 min, followed by 38 cycles of 94^{O}C for 15s, 58 ^{O}C for 15s, 72 ^{O}C for 30s with final extension step at 72 ^{O}C for 10min. The resultant band was excised and purified using MinElute gel purification kit (Qiagen). The purified fragment then was sent for sequencing (Source Bioscience, UK). The colony that contained the correct sequence was then picked and grown in LB broth plus ampicillin overnight. Plasmid DNA was then purified from cell lysates using PureYield Plasmid Midiprep system (Promega) according to the manufacturer's instructions (**Figure 2.3**). The copy number of plasmid DNA (with mitochondrial insert) was calculated based on the concentration obtained by Nanodrop measurement and stock of 4.1x10^{10} copies/µl was stored in aliquots at -20 until use.

Oocytes were fully denuded from cumulus cells using 0.3% hyaluronidase and then vortexed for 3min, washed and transferred individually in 2 µl of PBS/PVP 0.1% in PCR tube and stored in -20^{o}C until use. Fresh lysis buffer was prepared on the day of running PCR using 25 µl of 1M Tris-HCl, pH8; 25 µl Triton X-100; 2400 µl PCR water and 50 µl Proteinase K (NEB, P8107S). Lysis buffer was added in 6 µl to each single oocyte of 2 µl PBS/PVP and loaded on thermal cycler at 60^{o}C for 30min, 95 ^{o}C for 5min and hold at 4 ^{o}C.

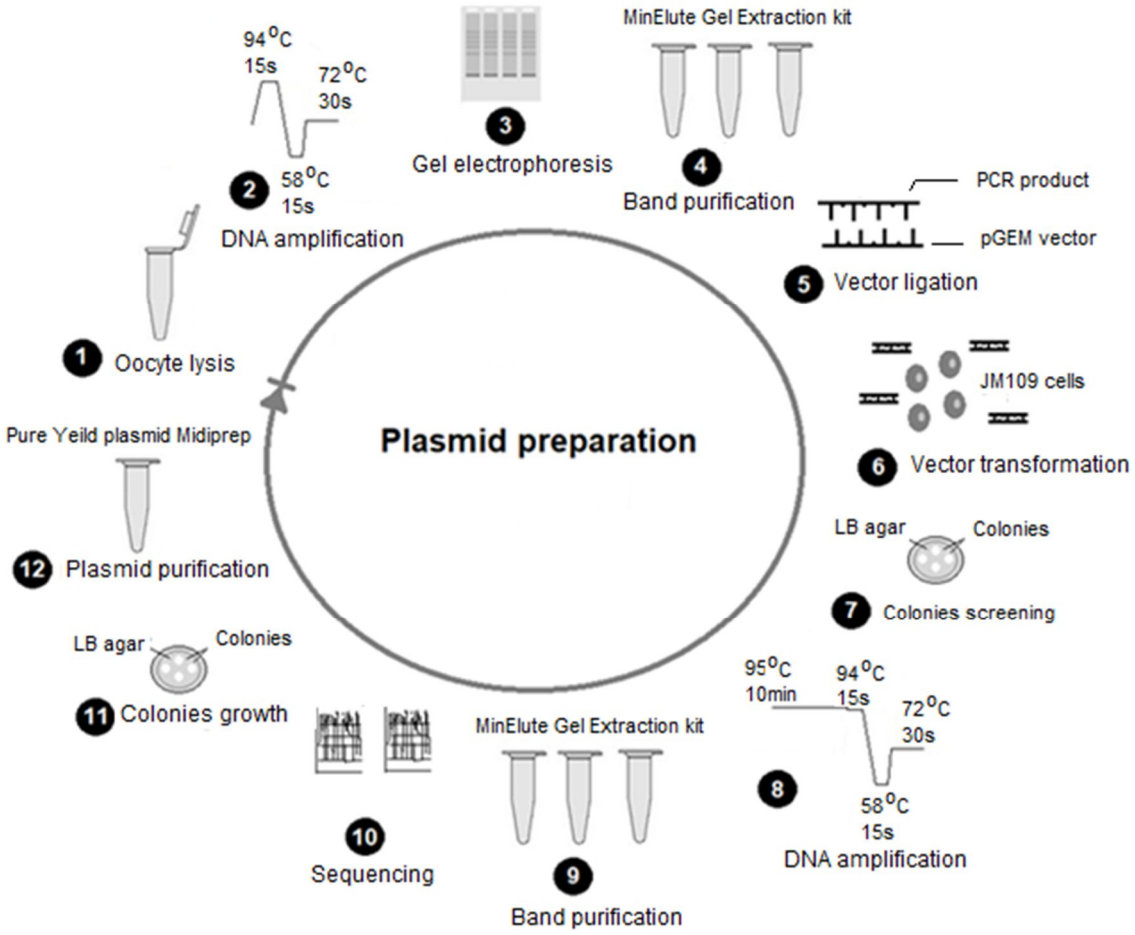

Figure 2.3. Schematic diagram illustrating the steps of plasmid preparation utilized for quantification of mtDNA copy number.

Absolute qPCR was performed to quantify mtDNA copy number. Reaction volume of 20 µl, contained 10 µl of 2x LightCycler 480 SYBR Green I Master mix (Roche), 0.2 µM of each forward and reverse primers B (**Table 2.1**), 5.7 µl of PCR water, and 3.5 µl of plasmid or lysed samples. All samples, blanks (only lysis buffer), and reagent controls (gDNA replaced with water) were used in duplicate. Reactions were performed using LightCycler®480 system (Roche, USA). Following a preincubation step at 95°C for 5 min followed by 45 cycles of (i) denaturation at 95°C for 10sec, (ii) annealing at 58°C for 15sec, (iii) extension at 72°C for 15s, with fluorescence signal acquisition at the end of each extension step; followed by melting curve analysis to check the specificity of PCR products. A five-fold serial dilution (10^3-10^7) of plasmid DNA was utilized to check the

linearity and efficiency of PCR amplification. The standard curve was also included in each run to allow absolute quantification of mtDNA copy number in samples. Four replicated experiments were performed, and each oocyte was analysed individually in duplicate in the same run for the detection of absolute mitochondrial copy number.

Calibration curves were based on a known concentration of prepared plasmid. Second derivative method was utilized for performing absolute quantitative analysis (Roche version 1.5 Manual). The efficiency of standard was calculated depending on the slope ($E=10^{(-1/Slope)}$) as previously mentioned by Pfaffl (2012). Amplification curve indicate fluorescent intensity and range of cycles for amplification of PCR products (Standard and samples) (**Figure 2.4A**). The standard curve with efficiency values 1.810-1.921, a slope of -3.5 to -3.8 and minimum error of 0.01 – 0.07 indicating the suitability of standard for use in quantification of mtDNA copy number (**Figure 2.4B**). Melting curves showed the downward of fluorescence in samples as they melt and the melting peak of samples (**Figure 2.4C, D**).

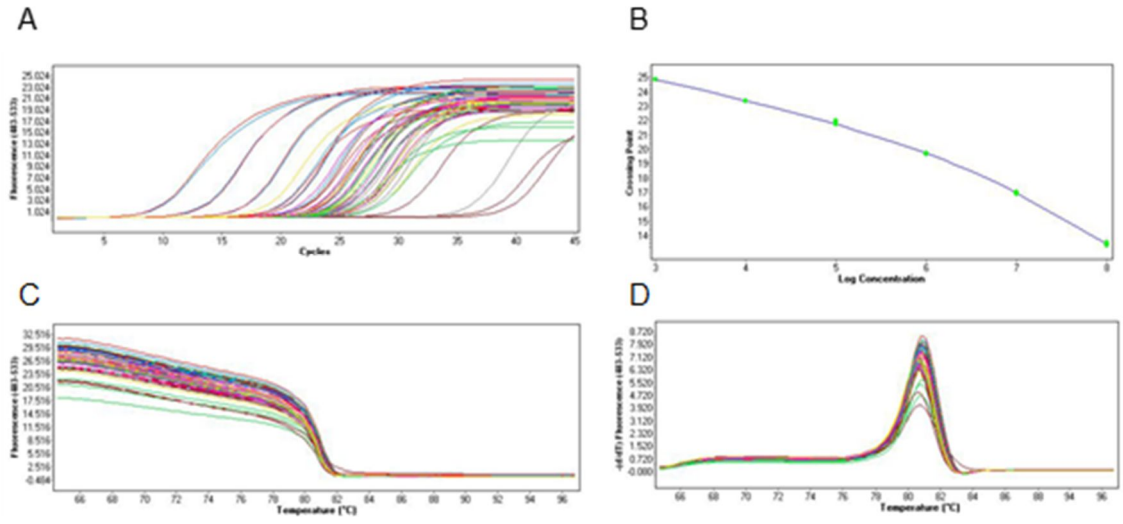

Figure 2.4. Amplification curve (A), Standard curve (B), downward trend of melting curves (C) and melting peak (D) for standards and samples utilized in quantification of mtDNA copy number.

2.2.6 Sperm preparation and *in vitro* fertilization

COCs were partially denuded by gentle pipetting and washed three times in HEPES-TALP medium containing 1,250 IU-1.5 mg ml^{-1} of Penicillin-streptomycin (P4333). COCs were then transferred to 4-well dishes with 400 µl each of Fert-TALP medium (Parrish et a., 1986, 2014), containing caffeine (1.39mmol/L) and 1250 IU-1.5 mg ml^{-1} of penicillin-streptomycin. Modified Bovipure gradient method (Nidacon International AB, Göthenborg, Sweden) was utilized in the preparation of sperms for *in vitro* fertilization (**Figure 2.5**).

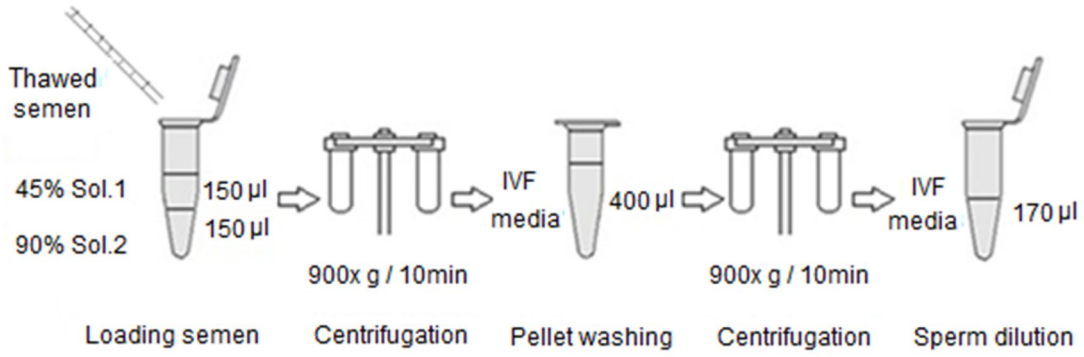

Figure 2.5. Schematic diagram illustrating the steps of Bovipure gradient for the preparation of bovine sperm for IVF.

Briefly, a solution of 90% (Solution-1) and 45% (Solution-2) utilizing Bovipure with Bovidiluent were prepared and pre-warmed before use. Then 150 µl solution-1 was placed in 0.6 µl microcentrifuge tube in the bottom layer and 150 µl solution-2 was uploaded in the upper layer. Semen was then placed on the top of the upper layer, and the tube was centrifuged at 900 x g for 10min. The resultant pellet was then resuspended in 400 µl of modified Fert-TALP medium and centrifuged at 900 x g for 10min. The resultant pellet was resuspended with 170 µl pre-incubated Fert-TALP medium and sperm counted and adjusted to the final concentration of 80 × 10^5 sperm/100 µl added to each well which containing 400 µl of Fert-TALP medium. In some specimens,

pronuclear formation was assessed post 8 h of fertilization utilizing Hoechst33342 staining according to Wang et al. (2008) (**Figure 2.6**).

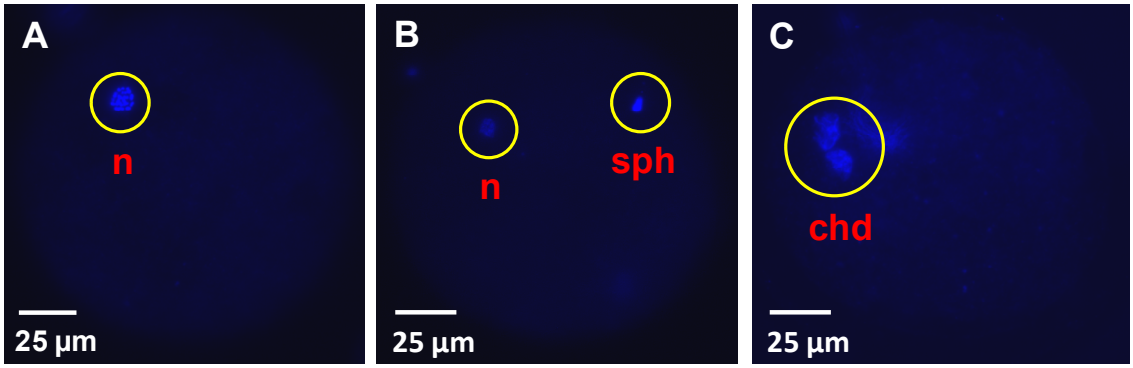

Figure 2.6. Bovine oocytes stained with Hoechst 33342 at 8 h post insemination in unfertilized oocyte with only nucleus (n) (A), penetrated oocyte with sperm head (sph) (B) and chromatin decondensation (chd) post fertilization (C).

2.2.7 *In vitro* embryo culture

At the end of fertilization, COCs were fully denuded from cumulus cells by pipetting and zygotes washed three times in h-SOF medium (Holms et al., 1999), containing HEPES but without gentamycin, myoinositol or BME amino acids (see Appendix III-H). Zygotes were then transferred to microdroplets of serum free m-SOF medium (without antibiotics-see Appendix III-G) in Nunc petri dish 60mm×15mm (size of microdroplet depended on number of zygotes; 1 zygote / 2 µl medium with maximum 40 µl droplet size). Zygotes were monitored for cleavage at D2 of culture or 46-48 h post-insemination. Medium were changed at D5 of culture. Blastocysts were monitored and assessed morphologically at D8 of culture (**Figure 2.7**) (see Appendix II-B). Also, Inner cell mass (ICM) and total cell numbers (TCC) were assessed at D8 by differential staining.

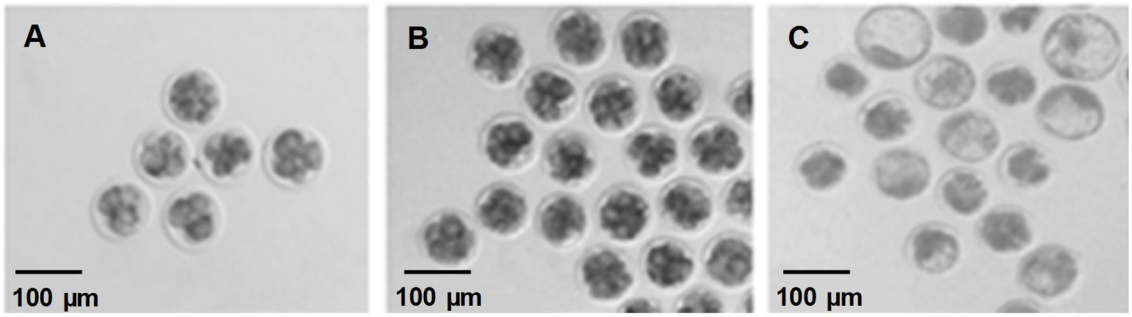

Figure 2.7. Cleaved zygotes at D2 (A) and D5 (B) with different stages and grades of bovine blastocysts (C) at D8 of *in vitro* culture.

2.2.8 Differential staining

A Modified version of the method by Thouas (2001) was used for staining blastocysts at D8 of culture. Solution-1 was prepared using 0.2%Triton-x (v/v) with PBS-PVP 0.1%, and Propidium iodide (PI) (P4170) was added so that the final concentration of PI was 125 µg ml^{-1}. Solution-2 was prepared using Hoechest33258 (B2883) and 4% paraformaldehyde in a dilution 1:1000 so that the final concentration of Hotchest 10 µg ml^{-1}. Briefly, blastocysts were transferred into solution-1 for 40s, after that embryos were washed thoroughly in SOF medium. Then blastocysts were transferred into solution-2 for 15-20min followed by three steps of washing with SOF medium. Blastocysts were then transferred in 2 µl medium and loaded with 3 µl fluoroshield (F6182) as a mounting medium on a slide, covered with coverslip, and sealed with nail varnish to fix it on the slide. Blastocysts were visualized and cells counted for both Inner cell mass (ICM) and total cell count (TCC) by epifluorescent microscope (Leica, DM4000B, Germany).

2.2.9 Effect of time and polar body during *in vitro* maturation

In order to investigate ATP content in relation to polar body extrusion in different time of *in vitro* maturation, total of 90 graded COCs were utilized in this study. ATP content was measured in fully denuded oocytes at the germinal vesicle stage or before *in vitro*

maturation. Also, ATP content for oocytes with or without polar body was assessed at 16 and 22 h of maturation.

2.2.10 Effect of DMSO administered for the last 6 h of *in vitro* maturation

In order to investigate the effect of DMSO at 0.03M (0.2 % v/v) during the last 6 h of *in vitro* maturation on ATP content and polar body extrusion, 151 graded COCs in five replicated experiments were utilized in this study. Oocytes were fully denuded at 22 h of *in vitro* maturation and visualized microscopically for polar body extrusion. ATP content was assessed for matured oocytes that extruded their first polar body at 22 h of maturation.

2.2.11 Effect of SNP and MG132 at 22 h of *in vitro* maturation

In order to determine the effect of SNP and/or MG132 on polar body extrusion and ATP content at 22 h of *in vitro* maturation, a total of 319 graded COCs in five replicated experiments were matured for the assessment of polar body, ATP content and mtDNA copy number. COCs were allocated into one of four groups; Control (DMSO), SNP, MG132 and SNP plus MG132 groups. It is necessary to dissolve MG132 in dimethyl sulfoxide (DMSO) before adding to culture. After 16 h of maturation, 10 μM MG132 was added to medium with final concentration of 0.03M (0.2 % v/v) DMSO and according to the manufacturer instructions. SNP was added to media throughout maturation in a final concentration of 10 μM and the DMSO was added during the last 6 h of maturation.

2.2.12 Effect of SNP and MG132 at different time points of maturation

In order to investigate the role of combinations SNP throughout maturation and MG132 in the last 6 h of maturation on nuclear maturation and mitochondrial activity at different time points, a total of 355 COCs and in five replicated experiment were utilized. Polar

body extrusion and ATP content were assessed at different time points of IVM (16 h, 19 h, 22 h, and 25 h).

2.2.13 Effect of SNP and/or MG132 on post fertilization development

In order to determine the effect of SNP throughout maturation and/or MG132 in the last 6 h of maturation on post fertilization development, an 8 replicated experiment with a total number of 673 graded COCs were utilized. These two factors were assessed in a 2 x 2 factorial arrangement. COCs were allocated to one of four groups; Control (DMSO), SNP, MG132, and SNP+MG132 groups. Cleavage was monitored at 46-48h post insemination and medium changed at D5 of IVC. Blastocysts were counted at D8 of IVC and evaluated morphologically under a light microscope for category and grade (Appendix-II). Inner cell mass and total cell count was assessed utilizing differential staining with Image-J software for cell counting.

2.2.14 Statistical analysis

Statistical analysis was undertaken to investigate the effect of SNP and MG132 on polar body extrusion, ATP content, mtDNA copy number and post fertilization development. Data were tested for homogeneity and distribution prior to statistical analyses. Generalized linear mixed models were performed to analyze ATP data and mtDNA copy number utilizing GenStat (GenStat software edition 17th, 2014). Polar body extrusion and post-fertilization development were analyzed utilizing logistic regression models as the data were binomially distributed. The results are presented with standard errors of the mean (SEM), and least significant differences, at P=0.05, were used to confirm differences between means. Different subscripts presented refer to differences between groups.

2.3 Results

2.3.1 Effect of time and polar body extrusion during *in vitro* maturation

In a pilot study to investigate the association of ATP content and polar body extrusion at different time points during maturation, a total of 90 denuded oocytes were utilised. A standard curve (**Figure 2.8A**) was generated. R^2 = 0.9975 indicating linearity and the suitability of standards for use in quantification of ATP.

ATP content was low (P=0.027) in germinal vesicle stage oocyte before *in vitro* maturation. Also, oocytes which did not extrude the polar body (PB) at 16 h of maturation had low (P<0.001) ATP content in comparison with extruded PBs and non-extruded PBs at 22 h of *in vitro* maturation (**Figure 2.8B**).

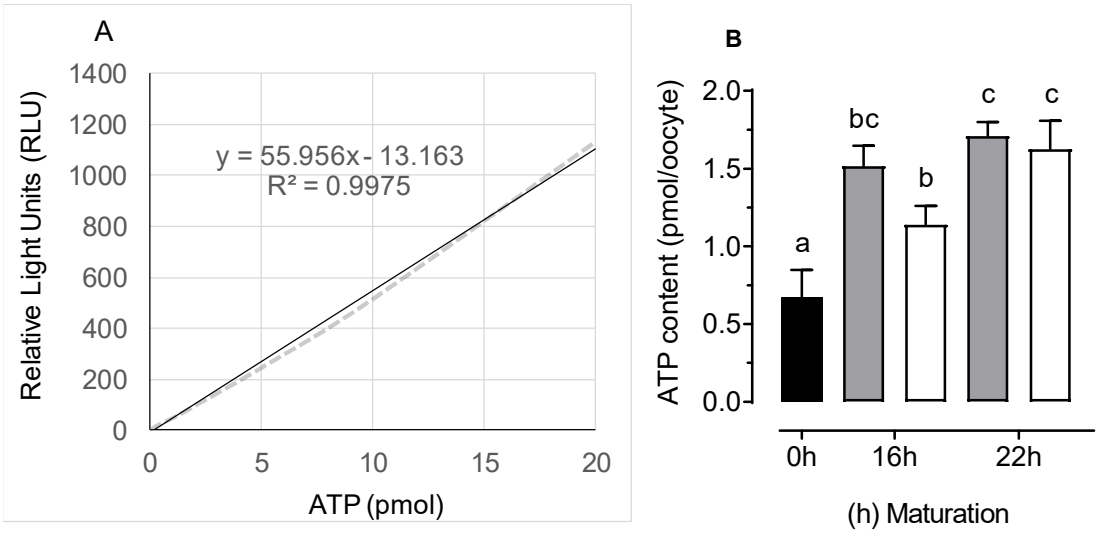

Figure 2.8. Standard curve (A), and ATP content in GV oocytes (black bar),16 and 22 h of *in vitro* maturation in oocytes that extruded a polar body (Grey bars) and oocytes with non-extruded polar bodies (white bars) (B). Means with different superscripts are different at P<0.05.

2.3.2 Effect of DMSO administered for the last 6 h of *in vitro* maturation

DMSO when added at 0.03M or 0.2 % (v/v) for the last 6 h of IVM had no effect on first polar body extrusion (**Figure 2.9**).

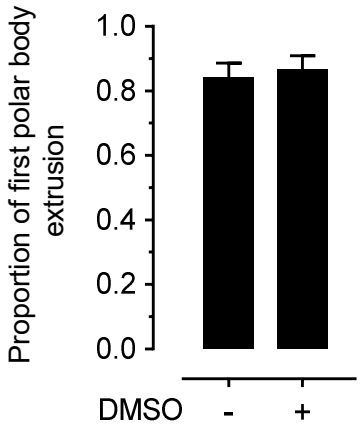

Figure 2.9. Proportion of first polar body extrusion in oocytes treated with and without DMSO during late *in vitro* maturation.

DMSO at the concentration used in this thesis also had no effect on ATP content (**Figure 2.10A, B**).

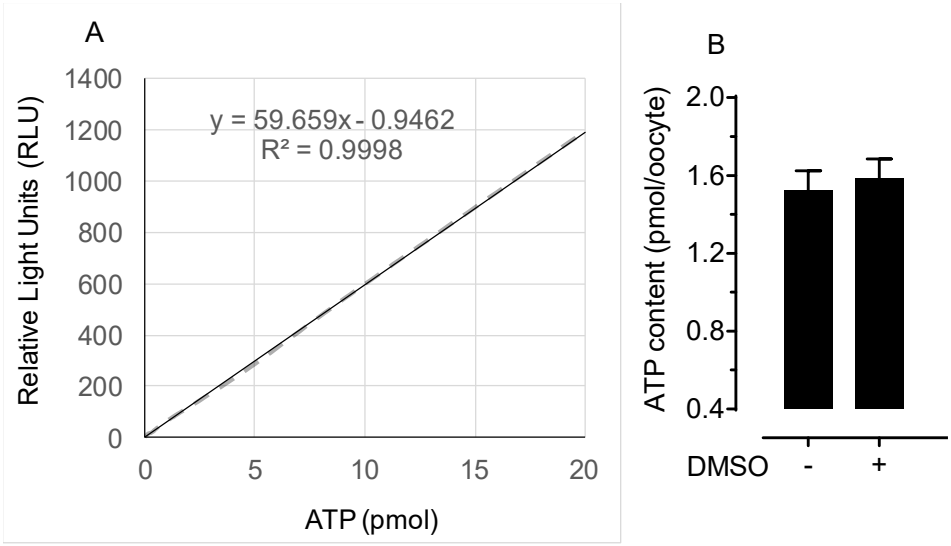

Figure 2.10. Standard curve and ATP content of oocytes cultured with and without DMSO.

2.3.3 Effect of SNP and or MG132 at 22 h of maturation

SNP and MG132 each significantly reduced first polar body extrusion in bovine oocytes at 22 h of IVM (P=0.013 and P=0.003 respectively) (**Figure 2.11**).

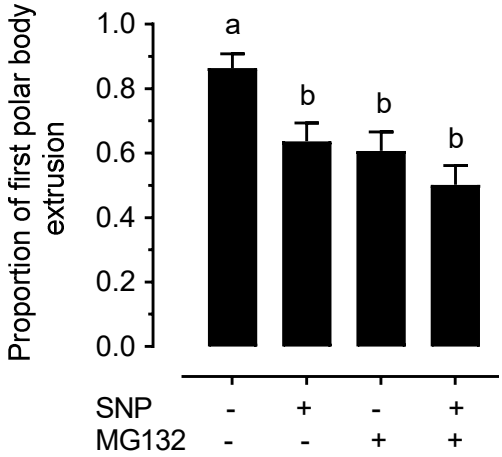

Figure 2.11. Proportion of first polar body extrusion at 22 post maturation in SNP and MG132 treated bovine COCs. Means with different superscripts are different at P<0.05.

With regard to ATP content in groups treated with SNP and/or MG132, data were generated from standard curve (**Figure 2.12A**). MG132 in the last 6 h of IVM reduced (P=0.003) ATP content in oocytes. However, SNP had no effect on ATP content when added throughout maturation (**Figure 2.12B**).

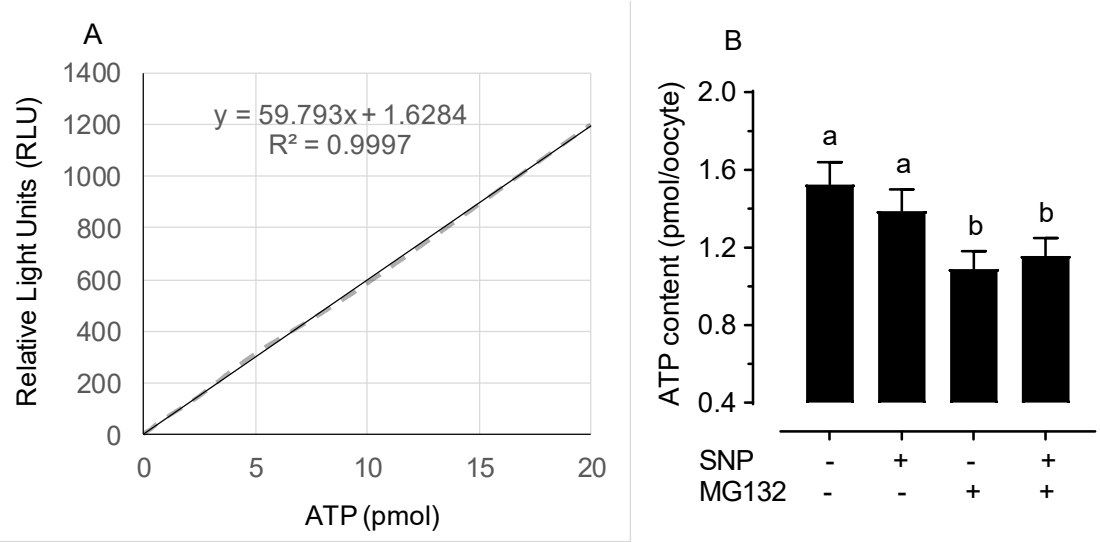

Figure 2.12. Standard curve (A), and ATP content in treated oocytes with SNP and/or MG132 during maturation (B). Means with different superscripts are different at P<0.05.

In terms of quantitative PCR, analysis was carried out to determine the effect of treatment with SNP and/or MG132 on mtDNA copy number. Data for mtDNA copy number was highly variable. Although, there was a numerical increase in mtDNA copy number in the combined SNP and MG132 group, there were no significant differences between treatments groups in terms of mtDNA copy number in denuded bovine oocytes at 22 h of *in vitro* maturation (**Figure 2.13**).

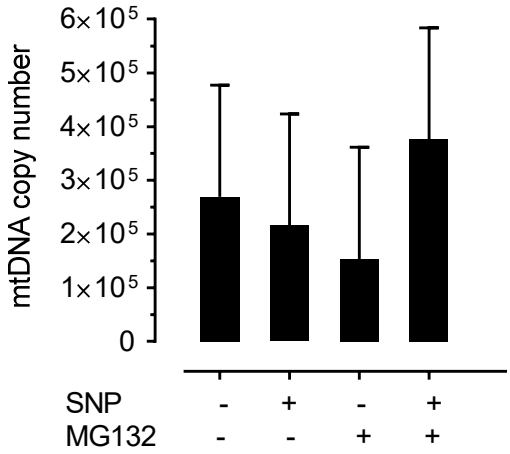

Figure 2.13. Absolute mitochondrial DNA copy number in bovine oocytes treated with SNP and MG132 during maturation.

2.3.4 Effect of SNP and MG132 at different time points of *in vitro* maturation

First polar body extrusion differed (P<0.001) with time of maturation. However, this proportion was reduced (P<0.001) post treatment with SNP plus MG132 (**Figure 2.14**). The least significant difference revealed that the proportion of polar body extrusion was significantly high at 19 h and 22 h but not 25 h of *in vitro* maturation. However, in comparison to the Control only 19 h time point did not differ between treatments.

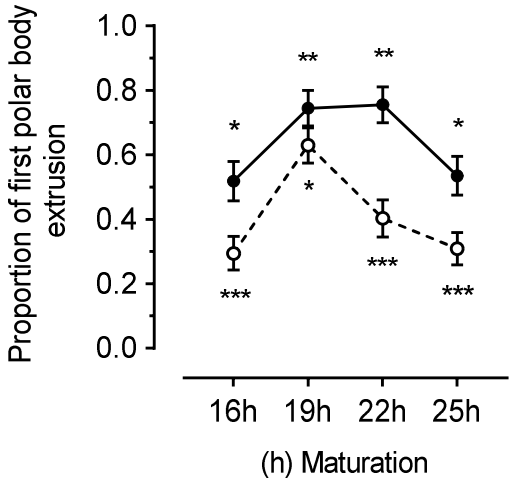

Figure 2.14. Proportion of polar body extrusion in ● Control and ○ SNP plus MG132 groups at different time points of *in vitro* maturation. Means with different superscripts are different at P<0.05. Experiment replicated five times.

ATP content increased (P=0.008) towards the end of maturation and reduced (P=0.002) post treatment with MG132. Also, in comparison with Controls counterpart, the reduction of ATP content was greater at 22 and 25 h of *in vitro* maturation post treatment with MG132 (**Figure 2.15**).

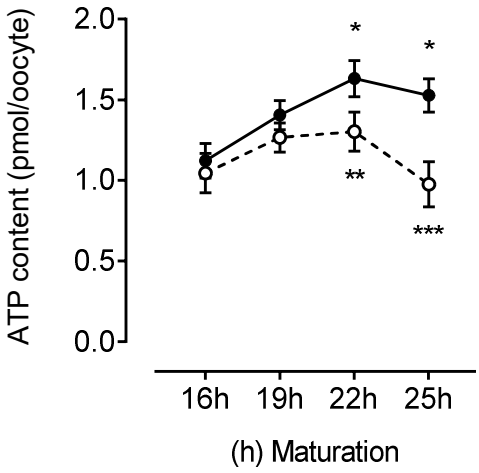

Figure 2.15. ATP content in bovine oocytes in ● Control and ○ SNP plus MG132 groups at different time points of *in vitro* maturation. Means with different superscripts are different at P<0.05. Experiment replicated five times.

2.3.5 Effect of SNP and MG132 on post fertilization development

Treatment with SNP or MG132 had no effect on proportion of cleaved zygotes at D2 of culture. However, this proportion was reduced (P=0.019) when SNP was combined with MG132 (**Figure 2.16A**). On other hand, MG132 alone or in combination with SNP reduced (P<0.001) proportion ≥4-cell embryos at D2. However, SNP during maturation had no effect on the proportion of well-developed embryos at D2 of culture (Figure **2.16B**).

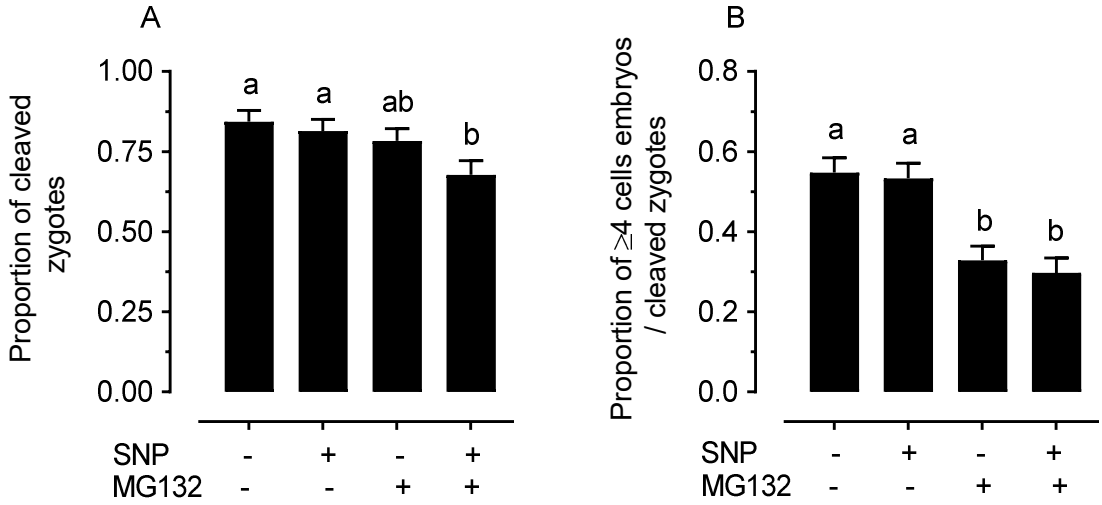

Figure 2.16. Proportion of cleaved zygotes (A) and ≥4-cell embryos at D2 of IVC. Means with different superscripts are different at P<0.05. Experiment replicated eight times.

MG132 reduced (P=0.001) the proportion of cleaved zygotes that reached the blastocyst stage at D8 of IVC. However, SNP had no effect on this proportion when applied individually (**Figure 2.17A**). On the other hand, neither SNP nor MG132 had an effect on the proportion of ≥4-cell embryos reaching blastocysts at D8 of IVC. However, there was an indication (P=0.07) that MG132 reduced this proportion (**Figure 2.17B**).

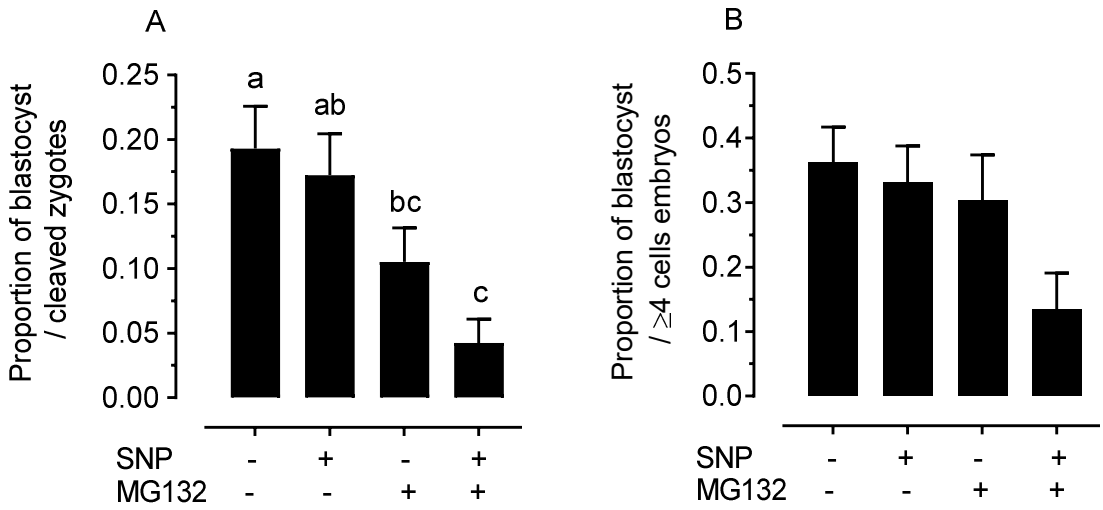

Figure 2.17. Blastocysts from cleaved zygotes (A) and ≥4-cell embryos at D8 of IVC post treatment of COCs with SNP and MG132 during maturation. Means with different superscripts are different at P<0.05. Experiment replicated eight times.

In relation to the quality of embryos, and in total 69 analyzed blastocysts, neither SNP nor MG132 had any effect on total cell number in early and late blastocysts at D8 of IVC. Also, the inner cell mass was not affected by SNP and or MG132 (**Figure 2.18A, B**).

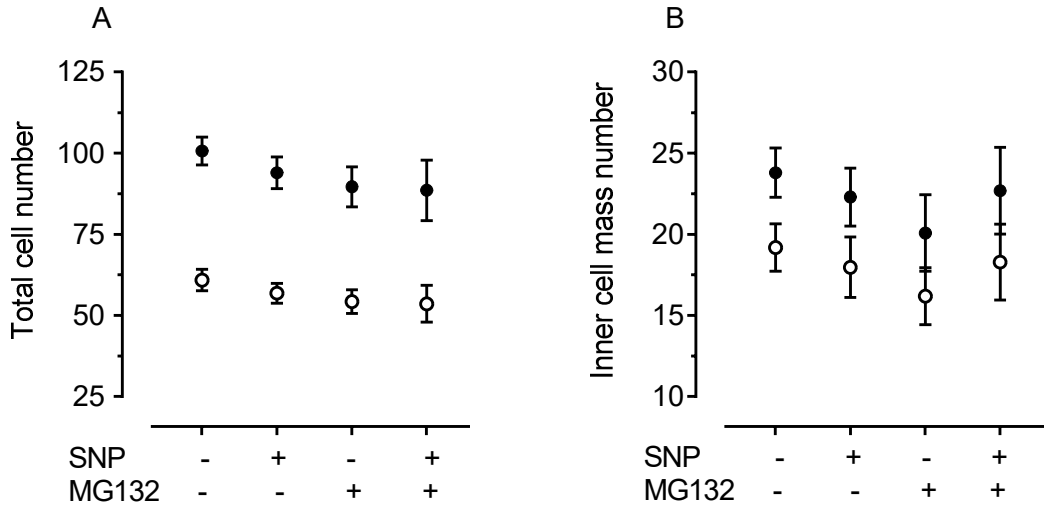

Figure 2.18. Total cell number (A) and Inner cell mass number (B) for ○ early and ● late blastocysts at D8 of *in vitro* culture and post treatment of COCs with SNP and or MG132 during *in vitro* maturation. Experiment replicated eight times (n=69).

Utilizing the modified technique of Thouas et al. (2001), it was possible to visualise the inner cell mass in only 51% of embryos, particularly those at the mid and late blastocyst stage (39% early, 63% mid and 67% late blastocyst). However, all harvested embryos were stained successfully with Hoechst33258 dye for total cell count (**Figure 2.19**).

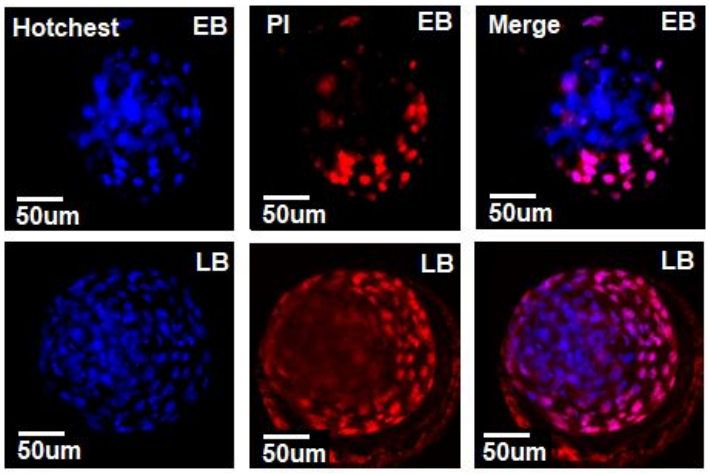

Figure 2.19. Differential staining of early (EB) and late blastocysts (LB) with Propidium iodide (PI) and Hoechst 33258 for total cell number and inner cell mass.

2.4 Discussion

The main objectives of the current study were to investigate the effect of the novel combination of SNP and MG132 on oocyte maturation and mitochondrial activity with respect to polar body extrusion, ATP content and mtDNA copy number, and on subsequent development of bovine zygotes post insemination. Also, to investigate polar body extrusion and ATP content at different time points of *in vitro* maturation. Moreover, finding out if DMSO affects polar body extrusion and ATP content when administered during late maturation. The series of experiments in this study showed that SNP and MG132 reduces polar body extrusion during maturation. Also, that SNP has no effect on ATP content, mtDNA copy number and post fertilization development of zygotes. However, MG132 reduces ATP content and post fertilization development when administered individually or in combination with SNP. Polar body extrusion and ATP

content increases during maturation but there was no effect of adding DMSO during late maturation on PBI extrusion and ATP content in comparison with the non-DMSO groups. Collectively, these findings indicate that both SNP and MG132 delay nuclear maturation at 22 h of maturation. Also, MG132 but not SNP reduces ATP content and subsequent embryonic development of bovine zygotes post fertilization.

2.4.1 Effect of time and polar body during *in vitro* maturation

With regards to ATP content, and in agreement with Nagano et al. (2006), ATP levels increased during maturation towards MII. Also, in accord with Stojkovic et al. (2001) no difference in ATP content between oocytes that extruded and non-extruded first polar body was observed at the end of maturation. However, ATP content differed between oocytes that extruded and did not extrude their first polar body at 16 h of maturation. As this is the first study, to our knowledge, to investigate differences in ATP content in relation to polar body extrusion earlier in maturation, low ATP content in oocytes that did not extrude their first polar body at 16 h of maturation might indicate low ATP demand in these oocytes.

2.4.2 Effect of DMSO administered for the last 6 h of maturation

DMSO in the current concentration (0.03M (0.2 % (v/v)) had no effect on polar body extrusion, and ATP content. Few studies have considered the effects of DMSO administered late during maturation on polar body extrusion and ATP content of bovine oocytes. However, DMSO affected oocyte maturation in a dose dependant manner but lower concentrations than 0.5% v/v had no effect on maturational events (Zhou et al., 2014 and Li et al., 2016). Therefore, administering DMSO for 6 h in late maturation had no effect on the nuclear maturation and ATP production at 22 h of maturation.

2.4.3 Effect of SNP and/or MG132 at 22 h of maturation

On the other hand, and in agreement with Dubey et al. (2011), 10 µM SNP negatively affected nuclear maturation and reduced polar body extrusion. The reduction of first polar body extrusion might indicate a role for both SNP and MG132 in delaying chromosomal segregation and the transition of oocyte from metaphase to anaphase. As reported previously, the delay of cell cycle progression in MI allows recovery of spindle formation and balanced chromosomal segregation with the possibility of multiple consequences for the delay in different cells (Potapova and Gorbsky, 2017). In the current experiment, low number of bovine oocytes that extruded first polar body post treatment with 10 µM SNP during maturation might be explained by the effect of SNP on sustaining adequate levels of cGMP, which will affect the resumption of meiosis and extrusion of first polar body in some oocytes.

On the other hand, MG132 added during late maturation reduces polar body extrusion separately or in combination with SNP. It has been proposed that treatment of bovine oocytes during late maturation with MG132 might reduce the ability of oocytes to reach MII, although effects were not significant (You et al. 2012a). Moreover, in another study the addition of MG132 at any time during maturation, even before polar body extrusion, prevented PB emission in rat oocytes. Furthermore, proteasome sensitive events occur shortly before the metaphase-anaphase transition (Josefsberg et al., 2000). It is possible that MG132 sustains high levels of cyclin B throughout by inhibition of its degradation by proteasome. Also, cyclin B degradation is an essential process for oocytes to overcome meiosis and transition to anaphase (Holloway et al., 1993; Josefsburg et al., 2000; Peters, 2002; Chang et al., 2003; Herbert et al., 2003; Parry et al., 2003 and Hyslop et al., 2004). In the current study, treatment of oocytes with 10 µM MG132 at 16 h might have delayed cell cycle progression in oocytes that were arrested in metaphase through abundance of cyclin B.

ATP content in the current study was reduced by MG132 but not SNP. Dalton et al. (2014) showed that cellular demand and consumption affects ATP level. Moreover, proteasomal degradation controls protein synthesis and degradation (Martinez-Vicente et al., 2005 and Rothman, 2010). Furthermore, 19s regulatory particle of proteasome and ubiquitination reactions are ATP dependant in the process of proteasomal degradation (Yi et al., 2009; Saez and Vilchez, 2014). In addition, the abundance of highly degradable regulatory proteins post treatment with MG132 (You et al., 2012a; Lane et al., 2013 and Sato et al., 2014) led us to the assumption that the decline of ATP levels in our experiments following treatment with MG132 during late maturation is related to the reduction of ATP demand for protein degradation.

In terms of mtDNA copy number, SNP and MG132 had no effect on copy number in bovine oocytes in the current study with great variation that could be related to the differences of the retrieved ovaries from animals of different age (Iwata et al., 2011; Takeo et al., 2013 and Kansaku et al. 2017).

Natriuretic peptide receptor 2 (NPR2) is activated by C- natriuretic peptide (CNP) for cGMP production in oocytes. Also, the LH surge decreases the level of CNP that limits the conversion of GDP to cGMP (Kawamura et al., 2011 and Sanchez and Smitz, 2012). Therefore, the presence of stimulatory hormones in the maturation medium might interfere with SNP to elevate cGMP which will be not sufficient for the increase of mtDNA copy number in oocyte.

On the other hand, MG132 has no effect on mitochondrial DNA copy number. Whereas treatment of young but not old bovine oocytes with MG132 increases mtDNA copy number (Kansaku et al., 2017). Therefore, the differences in the age of animal from which ovaries were collected could explain the variation of the copy number in the current study. However, further study in relation to the age of animal and treatment of oocytes with MG132 during late *in vitro* maturation is required.

2.4.4 Effect of SNP and MG132 at different time points during IVM

In control groups, PBI extrusion increased at 16 h and reached a peak at 19 to 22 h then declined again at 25 h post IVM. In similar tempo Van der Westerlaken et al. (1994) showed that the sharp increase of polar body extrusion is at 12 h with peak extrusion between 16-20 h post IVM. Also, it has been reported that degeneration of PBI occurs at ~30 – 40 h post IVM in some bovine oocytes (Hyttel et al., 1986). In the current study, around 50% of oocytes had no polar bodies at 16 h and the same percentage was observed at 25 h with peak extrusion of PBI between 19-22 h post IVM. This might indicate early degeneration of first polar body in oocytes which extruded their PB earlier (pre 16 h) during IVM. It seems that degradation of PBI is a continuous process during *in vitro* maturation.

SNP affects the proportion of PBI extrusion at 16 h before adding MG132. As previously mentioned, SNP at the concentration used in the current study affects nuclear maturation and delays cell cycle progression beyond MI and extrusion of PBI. It is possible that supplementation of media with SNP throughout maturation maintained levels of cGMP that affected nuclear maturation and delayed metaphase/ anaphase transition. On the other hand, 10^{-5} M MG132 during late IVM reduces polar body extrusion post 19 h post IVM. In agreement with previous studies (Holloway et al., 1993; Josefsburg et al., 2000; Peters, 2002; Chang et al., 2003; Herbert et al., 2003 and Parry et al., 2003), the inhibition of cyclin B1 degradation by MG132 arrested oocytes at metaphase and inhibited the transition to anaphase and extrusion of first polar body. Overall, both SNP and MG132 negatively affected the proportion of PBI extrusion in bovine oocyte. With regards to ATP content, SNP again had no effect on ATP content at 16 h post IVM. However, ATP content reduced post treatment with MG132 during late maturation and markedly so after 19 h IVM. The moderate reduction of intracellular ATP is a response to MG132 treatment inducing cell death in a time dependant manner (Huang et al., 2010).

It is possible that the reduction of cellular demand for protein synthesis and degradation is a response to MG132 treatment lowering ATP levels in oocytes.

2.4.5 Effect of SNP and or MG132 on post fertilization development

In accord with the findings of Soto et al. (2003), 10 μM SNP had no effect on cleavage or proportion of zygotes reaching the blastocyst stage in the current study. As mentioned earlier, the delay of oocyte progression beyond MI post treatment with SNP might recover spindle formation and chromosomal segregation and allow oocytes to become fertilized and proceed in development to the blastocyst stage. On the other hand, treatment of bovine oocytes with MG132 during late maturation, followed by incubation of gametes for 18 h during fertilization, reduces the developmental of zygotes reaching blastocyst stage. As mentioned earlier, You et al. (2012a) showed the beneficial effect of MG132 followed 8 h incubation of gametes for fertilization. A delay in gamete incubation post treatment of oocytes with MG132 reduces the development capacity of zygotes reaching the blastocyst stage. It was reported that highly polarized mitochondria in cumulus cells govern a variety of roles inside oocyte including ATP production and chromosomal segregation. Moreover, metabolically active mitochondria in cumulus cells are essential for oocyte growth and fertilization outcome (Dumesic et al., 2016 and Gorshinova et al., 2017). Furthermore, extracellular ATP is important in the process of porcine fertilization (Yi et al., 2009). It seems that cumulus cells play important roles during fertilization and reduction of ATP in oocytes might be an indication of the possible residual effect of MG132 in cumulus cells that leads to defective post fertilization development. Based on the series of experiments in this study, we propose that 10 μM MG132 reduced ATP content, and this reduction continued during the first hours of fertilization and disrupted post-fertilization development of zygotes resulting in low blastocysts yield.

2.5 Conclusions

This is the first detailed study to investigate the effects of SNP and MG132 during maturation on polar body extrusion, ATP content, mtDNA copy number and post fertilization development. The results of this study reveal that SNP reduces polar body extrusion but has no effect on ATP content, mtDNA copy number and post fertilization outcome. However, MG132 individually or in combination with SNP reduces polar body extrusion, ATP content during *in vitro* maturation and decreases post fertilization development of zygotes. It is suggested that the residual effect of 10 μM MG132 in cumulus cells during 18 h IVF reduces ATP content in oocytes negatively affects post fertilization outcomes. The next chapter will focus on the effect of utilizing different concentrations of MG132 during late maturation on subsequent development of zygotes and quality of blastocysts. Also, the effect of early denudation of COCs from cumulus cells post insemination (i.e. 8 h vs 18 h IVF) on post fertilization development and quality of blastocysts.

Chapter 3: Effect of MG132 dose during IVM and duration of exposure to semen during IVF on embryo development

3.1 Introduction

Reduction of protein degradation during late *in vitro* maturation might result in abundance of highly degradable regulatory proteins that prepare the matured oocyte for fertilization and subsequent embryonic development. However, MG132 might exhibit its beneficial effect at low concentrations or after a short period of sperm incubation with MG132 treated COCs during late maturation. As previously stated in Chapter 1 (Section 1.8), a short incubation period of oocytes with sperm for fertilization leads to high yields of blastocysts in bovine IVP (Ward et al., 2002 and Kochhar et al., 2003).

You et al. (2012a) showed that 10 µM MG132 during late maturation (16-22 h) followed by denudation of COCs 8 h post insemination resulted in abundance of glycolytic (e.g. GAPDH) and Anti-apoptotic (e.g. P4HB) proteins which had beneficial effects on oocytes and post fertilization development of bovine embryos. However, treatment of Yak COCs with 10 µM MG132 during late maturation (18 to 24 h) followed by denudation at 26 h post insemination had no effect on proportion of cleaved zygotes (Xiao et al., 2014). On the other hand, presence of 10 µM MG132 in the first hours of fertilization of bovine zygotes led to failure of pronuclear formation due to premature chromatin condensation that subsequently resulted in fertilization defects (Rawe et al., 2008). Moreover, 5 µM MG132 during fertilization affects development of mouse embryos through reduction of protein degradation which is an essential process in Maternal to Zygote Transition (MZT) post sperm penetration (Shin et al., 2010). It might be possible that MG132 at lower concentrations than 10 µM during late maturation is more beneficial for post fertilization development of bovine zygotes. On the other hand, a truncated period of IVF, and early denudation of bovine oocytes post treatment with 10 µM MG132, might eliminate the residual harmful effects of MG132 treatment on cumulus cells that leads to defective post fertilization development in conventional 18 h IVF. With regard to the MG132 solvent

DMSO, there are limited studies on its effects, when present during late oocyte maturation, on subsequent development of zygotes. Tsuzuki et al. (1998, 2000) showed that DMSO added at 50 µM during oocyte maturation, fertilization and embryo culture had a beneficial effect on the proportion bovine zygotes reaching the blastocyst stage. Also, Stinshoff et al. (2014) reported high blastocyst yields when bovine embryos were cultured in media supplemented with 0.01 M DMSO, and no effect of 0.03 M DMSO on embryonic development. However, supplementation of maturation medium with 0.4 M DMSO inhibited cumulus cell expansion and reduced nuclear maturation in porcine oocyte (Li et al., 2016), resulting in high rates of polar body deformity in mice (Zhou et al., 2014). Similarly, DMSO at >0.3 M in culture media reduced proliferation in mouse embryos (Kang et al., 2017) and blood lymphocytes (de Abreu Costa et al., 2017). The current study, therefore, sought to assess the effects of varying doses of MG132 (1 to 10 µM) incorporated during the final 6 h of IVM for differing periods of IVF, lasting either 18 or 8 h, on post-fertilisation development of bovine zygotes to the blastocysts stage.

3.2 Materials and methods

In vitro maturation, fertilization and culture media were prepared as described previously in Chapter 2 (Sections 2.2.2, 2.2.6 and 2.2.7). Briefly, oocytes were matured for 22 h in M199 maturation media. MG132, was applied at 16 h of IVM. Recovered oocytes were partially denuded, washed with HEPES- M199 media, and incubated with sperm in Fert-TALP media either for conventional (18 h) or short (8 h) periods. The resultant zygotes were fully denuded and transferred to SOF culture media in microdroplets covered with mineral oil. Zygotes were monitored for cleavage at D2 of culture, around 46 - 48 h post sperm incubation. Cleaved zygotes were then transferred to fresh culture media. Embryos were subsequently transferred to fresh media on D5 of IVC. Blastocysts were harvested at D8 of IVC, graded and stained for cell counting as previously described in Chapter 2 (Section 2.2.8)

3.2.1 Effect of MG132 at different concentrations during late IVM on post fertilization development

Different stock concentrations (0.5, 2.5, and 5mM) of MG132 were prepared, using DMSO as a solvent (final concentration of DMSO in media was 0.2% v/v and according to the manufacturer's instructions), and added in 2 µl volume to the medium so that the final concentration of MG132 was 1, 5, and 10 µM. Five replicated experiments with a total of 447 Grade 1 and 2 COCs were undertaken. COCs were randomly allocated to one of four treatment groups; Control (DMSO) (0 µM), 1 µM, 5 µM, and 10 µM. Proportion cleaved, and stage of development were assessed on D2 of IVC (46-48h post insemination). Also, blastocyst development and quality were evaluated by morphological grading and differential staining (see Chapter 2 Section 2.2.8) on D8 of IVC.

3.2.2 Effect of DMSO on post fertilization outcome

To determine the effect of 0.03M (0.2 % v/v) DMSO on post fertilization development of zygotes, four replicated experiments used 347 Grade 1 and 2 COCs. COCs were allocated to one of four treatment groups: without DMSO – 8 h, with DMSO - 8 h, without DMSO – 18 h and with DMSO – 18 h. 0.03 M (0.2% v/v) DMSO was applied during the last 6 h of IVM. Fertilization were performed as described previously in Chapter 2 (Section 2.2.6) except the timing for 18 and 8 h incubation period of sperm with partially denuded COCs.

3.2.3 Effect of MG132 followed by 8 h or 18 h IVF on embryo development

This study was undertaken to investigate the effect of different durations of IVF (conventional 18h vs 8 h) on subsequent embryo development post treatment of bovine oocytes with 10 µM MG132 during the final 6 h of IVM. Four replicated experiments utilising 396 Grade 1 and 2 COCs were undertaken. COCs were randomly allocated to

one of four treatment groups: Control (DMSO) - 8 h, MG132 - 8 h, Control (DMSO) -18 h and MG132 -18 h). Briefly, after 6 h treatment with MG132 during late IVM, COCs were partially denuded from cumulus cells followed by incubation with sperm for 18 h or 8 h for fertilization and as previously described in Chapter 2 (Section 2.2.6). Putative zygotes were then fully denuded, washed with hSOF medium, cultured in serum free SOF medium in micro drops covered with oil as discussed previously in Chapter 2 (Section 2.2.7).

3.2.4 Statistical analyses

Statistical analysis was performed to explore the association of different MG132 concentrations, also the effect of early denudation of bovine oocytes post treatment with 10 μM MG132 during late maturation, on post fertilization development of bovine zygotes. Moreover, the effect of DMSO on post fertilization outcomes was also assessed. Statistical analyses were performed using the GenStat statistical package (17th Edition, VSN International, 2011). All proportion data were analysed using generalized linear regression models that assumed binomial errors and used logit-link functions. Analyses of cell number (inner cell mass and trophectoderm) used generalized linear regression models but assumed Poison errors and used log-link functions. Residual plots confirmed that data were normally distributed and homogeneous. Data are presented as predicted means and standard error of these means (s.e.m). In each case, differences between individual means were established using Least Significant Differences (LSD) derived from residual errors of the statistical models. Differences were considered significant at $P<0.05$.

3.3 Results

3.3.1 Effect of different concentrations of MG132 during late maturation on post fertilization development

MG132 included during the final 6 h of maturation at concentrations between 1 and 10 μM had no significant effect on the proportion of D2 cleaved zygotes of total oocytes (**Figure 3.1**). MG132 included at 1 μM and 5 μM during late maturation had no significant effect on embryo development (cell number) by D2 following insemination (**Figure 3.2**).

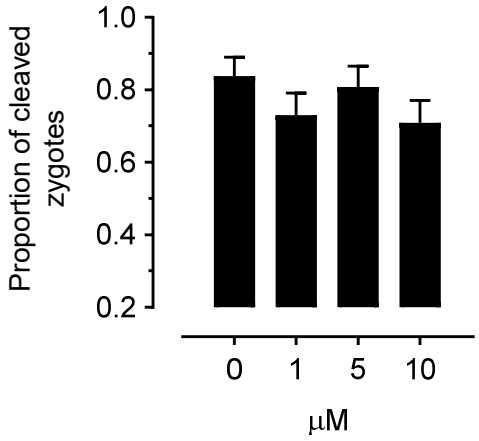

Figure 3.1. Proportion of D2 cleaved zygotes following treatment with different concentrations of MG132. Experiment replicated five times.

However, 10 μM MG132 reduced the proportion of 4-cell (P=0.058) and >4-cell embryos (P=0.03) in D2 of culture (**Figure 3.2**).

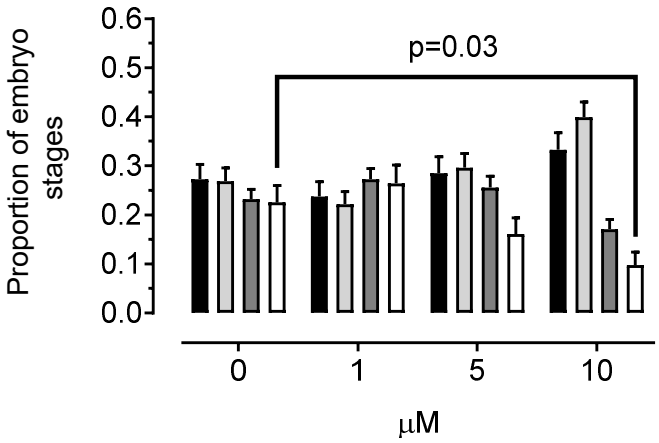

Figure 3.2. Distribution of bovine embryo stages on D2 following insemination of oocytes matured with different concentrations of MG132. ■ 2-cell; ■ 3-cell; ■ 4-cell; □ >4-cell. Experiment replicated five times.

The proportion of ≥4-cell embryos from oocytes and cleaved zygotes was reduced (P=0.04) with 10 μM MG132 during late maturation (**Figure 3.3A, B**).

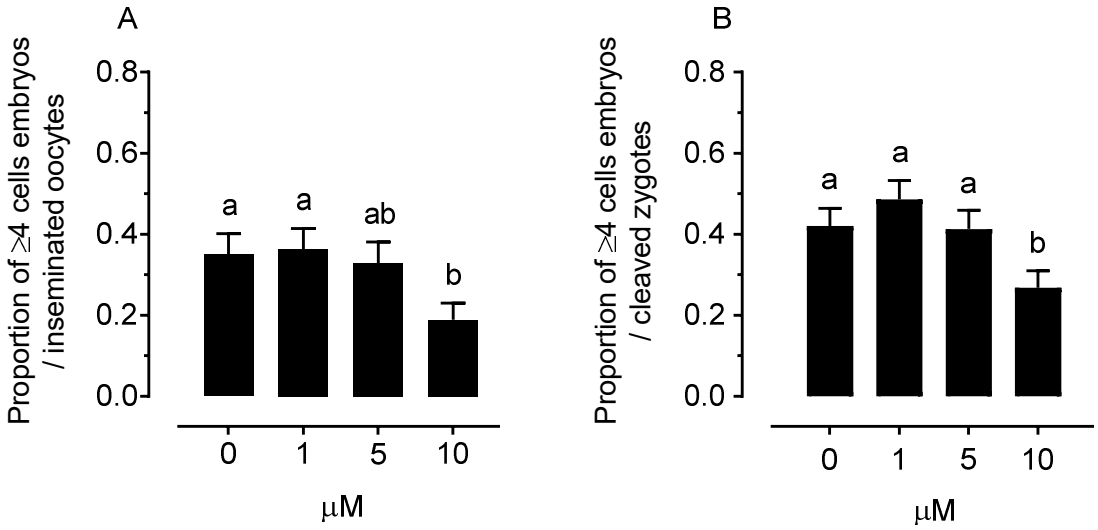

Figure 3.3. Proportion ≥4-cell embryos from oocytes (A) and of D2 cleaved zygotes (B) following the treatment of COCs with different concentrations of MG132. Means with different superscripts are different at P<0.05. Experiment replicated five times.

MG132 dose had no significant effect on the proportion of blastocysts from inseminated oocytes, although numerically there was an indication that 10 μM MG132 added during late maturation reduced the proportion of D8 blastocysts (**Figure 3.4**).

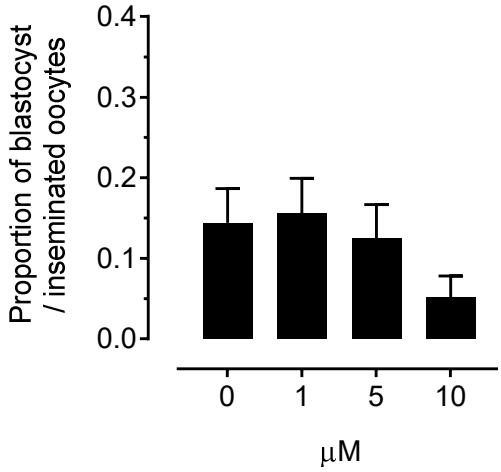

Figure 3.4. Proportion of blastocysts from inseminated oocytes at D8 of culture following maturation of oocytes with different concentrations of MG132. Experiment replicated five times.

Similarly, there was an indication that the proportion of blastocysts from D2 cleaved zygotes and ≥ 4-cell embryos was also reduced when oocytes were matured with 10 μM MG132, but again this did reach statistical significance (**Figure 3.5**).

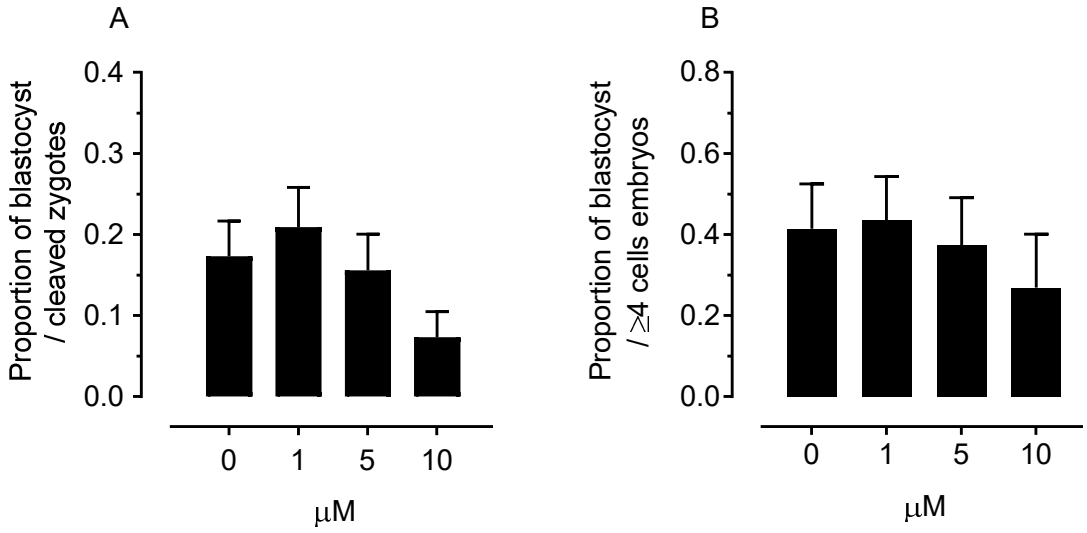

Figure 3.5. Proportion of blastocysts at D8 of culture from cleaved zygotes (A) and ≥4-cell embryos at D2 of culture (B) following insemination of oocytes matured with different concentrations of MG132. Experiment replicated five times.

In total 53 analysed blastocysts, MG132 dose had no effect on the total number of cells in early and late blastocysts at D8 of culture (**Figure 3.6A**), nor on the number of cells within the inner cell mass (**Figure 3.6B**).

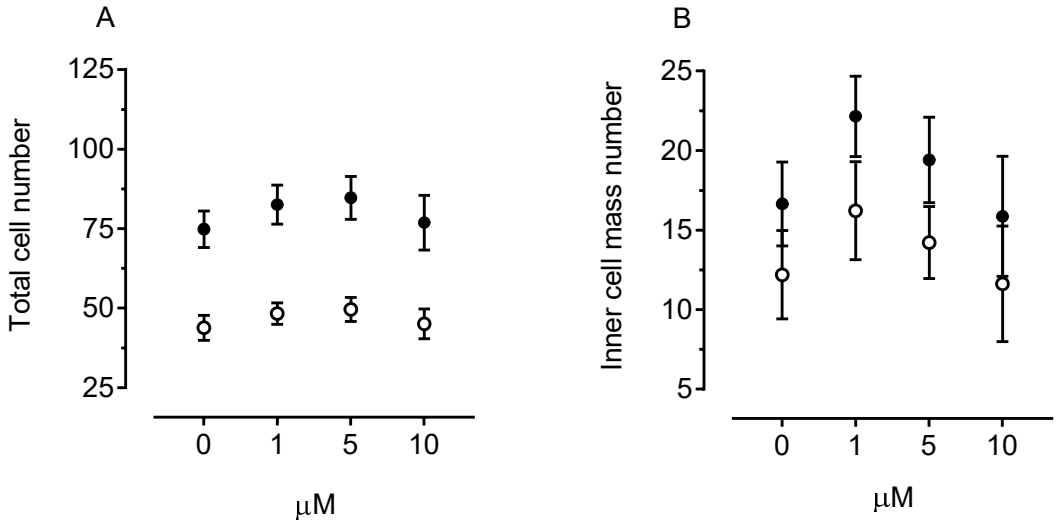

Figure 3.6. Total cell (A) and Inner cell mass number (B) at D8 of culture following insemination of oocytes matured with different concentrations of MG132 in ○ early and in ● late blastocysts. Experiment replicated five times (n=53).

3.3.2 Effect of DMSO post oocyte denudation at 8 h or 18 h following sperm incubation on subsequent embryo development

In a pilot study to investigate the effect of DMSO on post-fertilization outcome, a total of 347 COCs were analysed. DMSO has no significant effect on proportion cleaved zygotes on D2 of culture. However, a short incubation period of sperm with oocytes for 8 h reduced (P=0.003) this proportion (**Figure 3.7A**). Also, the proportion ≥4-cell embryos on D2 of culture was not affected by DMSO, but early denudation of oocytes 8 h post insemination increased (P<0.001) this proportion (**Figure 3.7B**). On other hand, there was no effect of either DMSO or duration of IVF on the proportion blastocysts of inseminated oocytes (**Figure 3.7C**). Moreover, the proportion D8 blastocysts was not affected by DMSO, but there was an indication that a short incubation period (8 h) of

sperm with oocytes increases (P=0.056) the proportion D8 blastocysts of cleaved zygotes (**Figure 3.7D**).

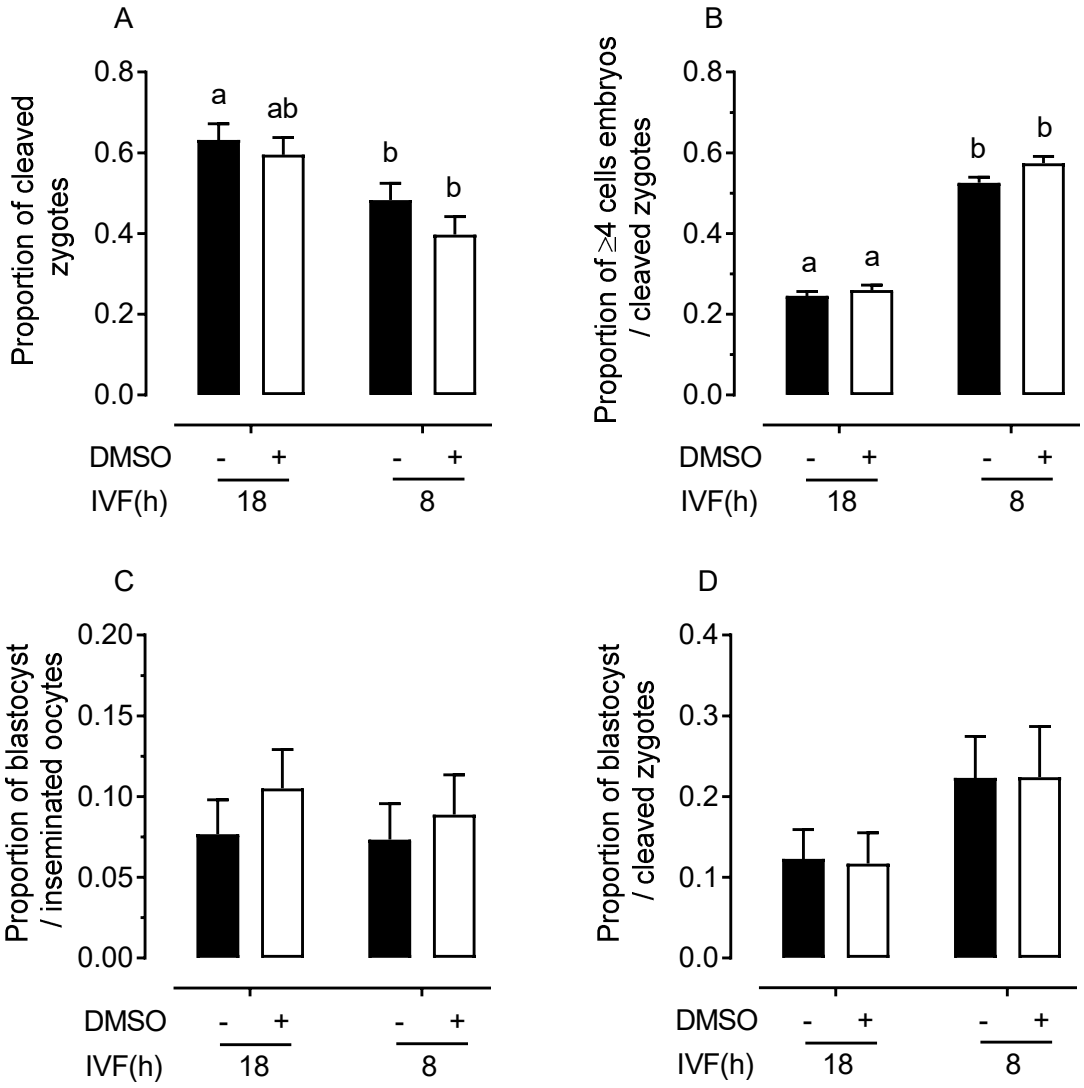

Figure 3.7. Effect of DMSO and timing of IVF on proportion of cleaved zygotes at D2 (A), ≥4-cells embryos from cleaved zygotes (B), D8 blastocysts from inseminated oocytes (C), and D2 cleaved zygotes (D), without (black bars) and with (white bars) DMSO. Means with different superscripts are different at P<0.05. Experiment replicated four times. Experiment replicated four times.

On the other hand, and in total 30 harvested blastocysts, DMSO had no effect on total cell number and Inner cell mass number at D8 of IVC (**Table-3.1 a, b**).

Table 3.1a Predicted mean values (Mean±SEM) for Total cell number in early and late blastocysts for 18 and 8 h IVF regime in treated groups with or without DMSO at late *in vitro* maturation. Experiment replicated four times.

	Total cell number			
	18 h		8 h	
	Early blastocyst	Late blastocyst	Early	Late blastocyst
-DMSO	60.0± 4.97	91.5± 6.75	62.4± 6.45	93.8±8.28
+DMSO	51.5± 5.51	82.9±6.76	67.7± 7.66	99.1±9.77

Table 3.1b Predicted mean values (Mean±SEM) for Inner cell mass in early and late blastocysts for 18 and 8 h IVF regimes in treated groups with or without DMSO at late *in vitro* maturation. Experiment replicated four times.

	Inner cell mass			
	18 h		8 h	
	Early blastocyst	Late blastocyst	Early blastocyst	Late blastocyst
-DMSO	13.0±0.76	15.0±0.88	16.8±0.50	18.8±0.92
+DMSO	14.7±0.71	16.7±1.11	18.4±1.34	20.4±1.19

3.3.3 Effect of MG132 post oocyte denudation at 8 h or 18 h following sperm incubation on subsequent embryo development

Relative to 18 h Control (i.e. no MG132), the proportion of cleaved zygotes was reduced (P<0.001) post 8 h IVF in the absence but not the presence of 10 μM MG132 administered during late maturation (P=0.013) (**Figure 3.8**).

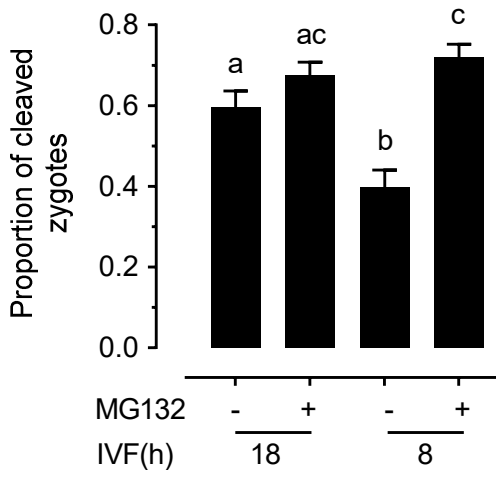

Figure 3.8. Proportion of cleaved zygotes post treatment with 10 μM MG132 during late maturation followed by two different IVF durations. Means with different superscripts are different at P<0.05. Experiment replicated four times.

The proportion of 2-cell embryo from total cleaved embryos at D2 of culture was greater (P=0.004) for 18 vs 8 h IVF and when MG132 was present rather than absent during IVM (P=0.002) (**Figure 3.9A**). On the other hand, the proportion of 3-cell embryos was not affected by IVF duration or MG132 treatment during late maturation (**Figure 3.9B**). There was an interaction (P=0.003) between IVF duration and MG132 treatment for the proportion of 4-cell embryos which indicated a negative effect of MG132 at 18 h but a positive effect at 8 h (**Figure 3.9C**). In contrast to the proportion of 2-cell embryos, the proportion of >4-cell embryos from cleaved zygotes at D2 was greater (P<0.001) for 8 vs 18 h and in the absence rather than the presence of MG132 during IVM (P=0.001) (**Figure 3.9D**).

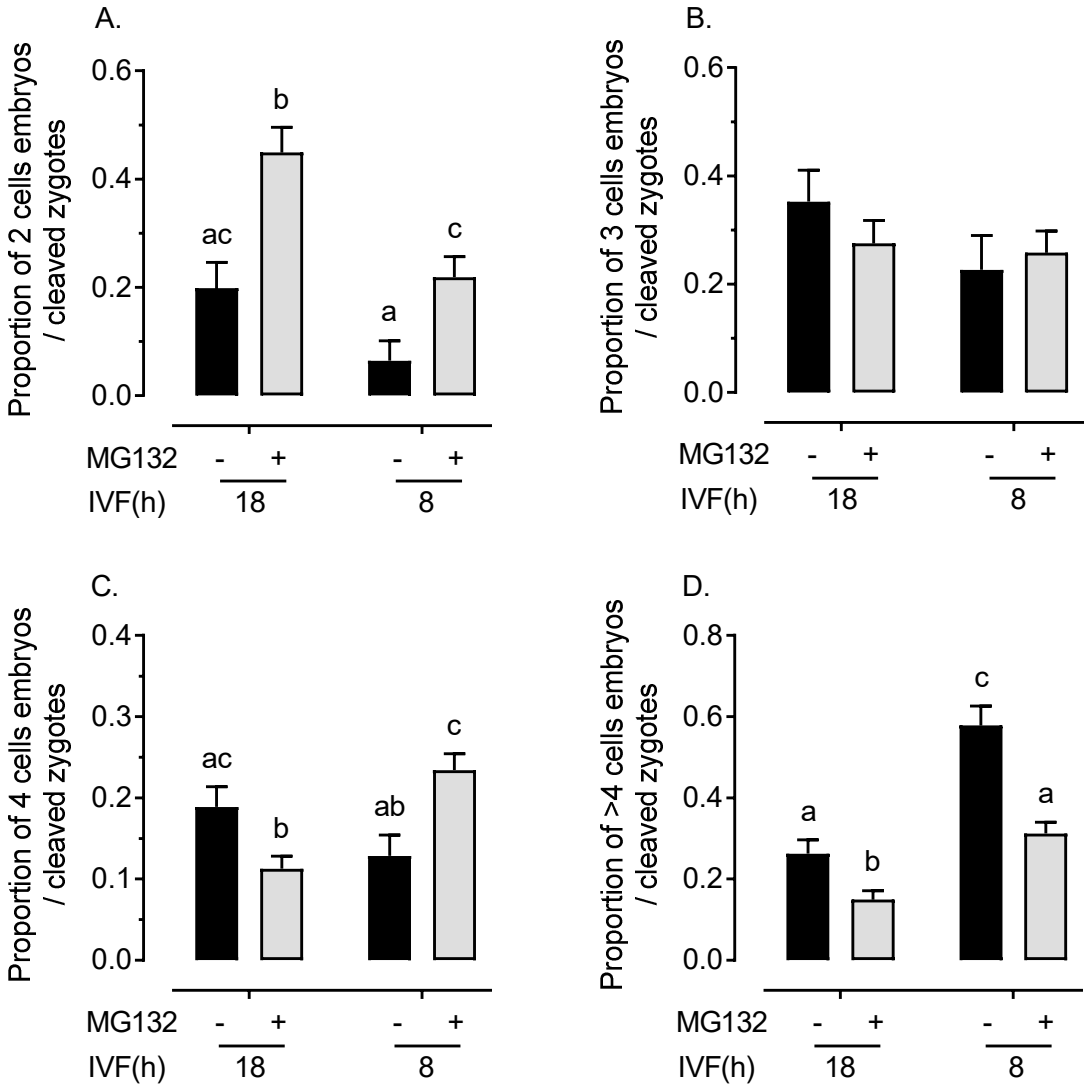

Figure 3.9. Bovine embryos from cleaved zygotes at D2 of culture following 18 h vs 8 h IVF for oocytes matured with 0 (black bars) or 10 μM (grey bars) MG132. Means with different superscripts are different at P<0.05. Experiment replicated four times.

There was an interaction (P=0.002) between IVF duration and MG132 on the proportion ≥4-cell embryos from inseminated oocytes which indicted a positive effect of 8 compared to 18 h IVF in the presence of MG132 (**Figure 3.10A**). Following on from data presented in **Figure 3.9**, the proportion ≥4-cell embryos from cleaved zygotes was greater (P<0.001) for 8 vs 18 h IVF, but was reduced (P=0.002) in the presence of MG132 (**Figure 3.10B**).

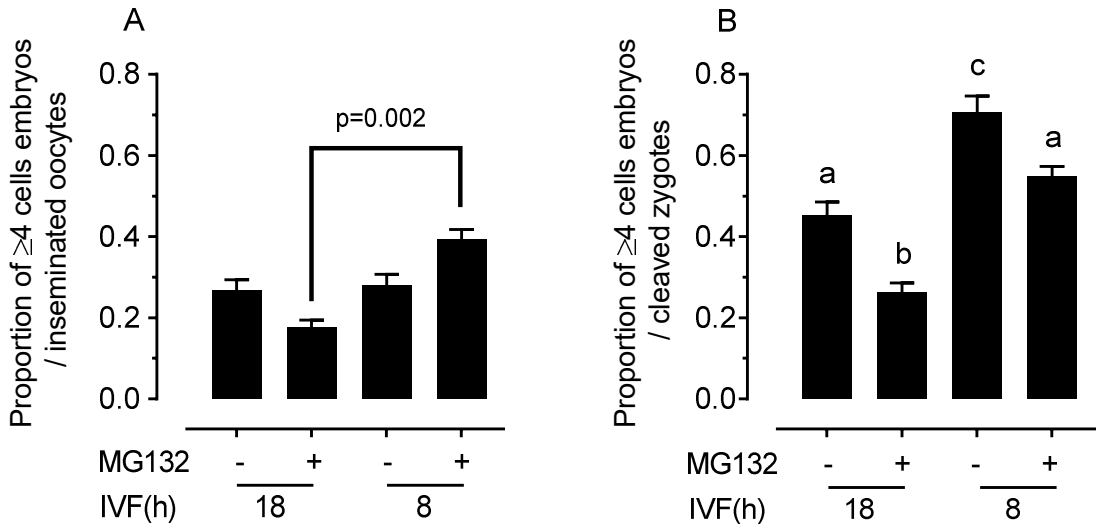

Figure 3.10. Proportion of ≥ 4-cell embryos from inseminated oocytes (A) and cleaved zygotes (B) at D2 of culture following 18 h vs 8 h IVF for oocytes matured with 0 or 10 µM MG132. Means with different superscripts are different at P<0.05. Experiment replicated four times.

The proportion of blastocysts produced from inseminated oocytes was greater (P=0.007) for 8 vs 18 h IVF in the treatment groups (**Figure 3.11**). There was also an indication that MG132 treatment during IVM followed by 8 h IVF increased the proportion blastocysts of inseminated oocytes, but this was not statistically significant (P=0.061).

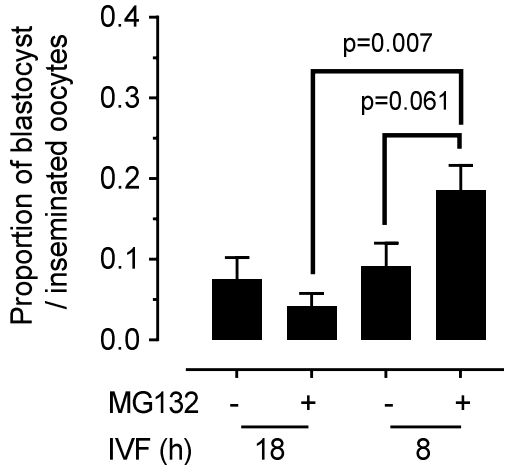

Figure 3.11. Proportion of blastocysts from inseminated oocytes at D8 of culture following 18 h vs 8 h IVF of oocytes matured with either 0 or 10 µM MG132. Experiment replicated four times.

MG132 had no effect on the blastocyst proportion of cleaved zygotes at D2 of culture, but this measure of development was greater (P=0.004) following 8 vs 18 h IVF in the treatment groups (**Figure 3.12A**). There was no effect of treatment on the proportion of blastocysts from ≥4-cell embryos (**Figure 3.12B**).

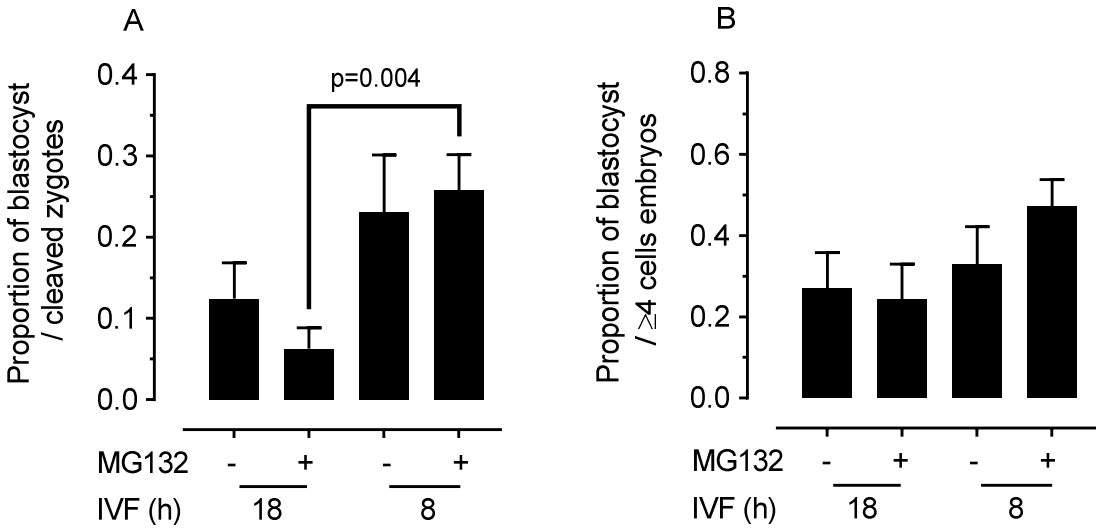

Figure 3.12. Proportion of blastocyst from cleaved zygotes (A) and of ≥4-cell embryos (B) at D8 of culture following 18 h vs 8 h IVF for COCs matured with either 0 or 10 μM MG132. Experiment replicated four times.

In total 40 analysed blastocysts, Total cell number differed (P<0.001) between early and late blastocysts, but neither MG132 treatment during IVM or duration of IVF affected total cell number and number of ICM cells in D8 blastocysts (**Figure 3.13**).

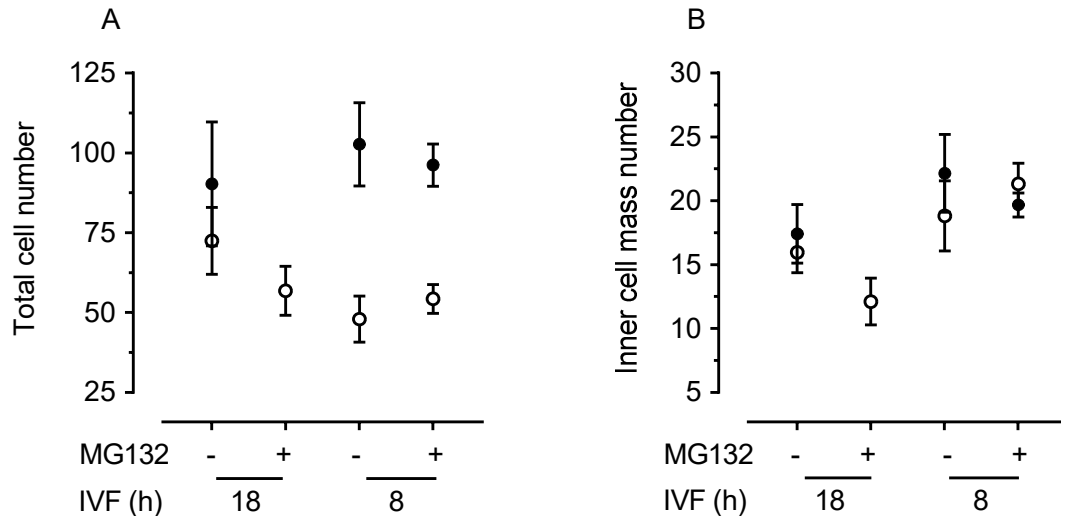

Figure 3.13. Total cell number (A) and inner cell mass (B) at D8 of culture following 18 h vs 8 h IVF for oocytes matured with either 0 or 10 µM MG132. Date presented in ○ early and ● late blastocysts. Experiment replicated four times (n=40).

3.4 Discussion

The current study was undertaken to clarify the negative results of utilizing 10 µM MG132 in late maturation followed by 18 h IVF on post fertilization development of bovine zygotes observed in Chapter 2. This study revealed that MG132 at low concentrations (1 and 5 µM) has no effect on post fertilization development of zygotes, although 10 µM appears to be cytotoxic. Also, a truncated incubation period of gametes for fertilization following treatment of oocytes with 10 µM MG132 during late maturation reverses the negative effect of MG132 observed with conventional 18 h IVF. In these experiments, the inclusion of the solvent DMSO had no effect on post fertilization development.

3.4.1 MG132 concentration during late maturation

The results of this experiment indicate that MG132 at concentration of 1 and 5 µM during late maturation has no effect on cleavage and the proportion of zygotes that reached the blastocyst stage. This finding supports evidence from previous studies (You et al., 2012a and Xiao et al., 2014). Consistent with the finding in previous experiments, treatment of

oocytes during last 6 h of maturation with 10 µM of MG132 suppresses post fertilization development of zygotes and reduces blastocyst yield. It is likely that the prolonged residual effect of 10 µM MG132 administered during late maturation leads to a reduction of ATP content in oocytes (as demonstrated in Chapter 2; Section 2.3.3) and cyclin B degradation. ATP supply sustains Ca^{+} oscillations (Dumollard et al., 2008; Campbell and Swann, 2006), which in turn promotes cyclin B degradation and helps zygotes to overcome MII arrest (Marangos and Caroll, 2004). However, an extended insufficient supply of ATP during the first hours of fertilization leads to failure of endoplasmic reticulum (ER) Ca^{+} refill and post fertilization defects (Takahashi et al., 2000; Liu et al., 2001; Gordo et al., 2002; Igarashi et al., 2005). Based on these findings, and that 10 µM MG132 followed by 18 h IVF reduces ATP content and degradation of cyclin B, one can hypothesize that this would impair post-fertilization development.

3.4.2. 18 h vs 8 h IVF

In contrast with Long et al., (1994) and Rehman et al., (1994), It has been shown that short incubation period of gametes *in vitro* reduces fertilization rate in bovine (Ward et al., 2002) and human (Barraud-Lange et al., 2008). Also, It has been demonstrated that truncated IVF systems (i.e. 5-15 h) reduced polyspermy (Sumantri et al., 1997; Kochhar et al., 2003 and Iwata et al., 2008) and accumulation of harmful free radicals (Enkhmaa et al., 2009 and Lopes et al., 2010) leading to zygotes with greater developmental competence. Our results showed that a short incubation period (8 h) of gametes reduces fertilization (around 40 %) but increases the blastocyst proportion from cleaved zygotes.

The increase proportion of blastocysts from cleaved zygotes at D2 post 8 h IVF is related to the reduction in the proportion of cleaved zygotes. This adds evidence to previous research (Ward et al., 2002 and Kochhar et al., 2003) that the delay in the incubation of sperm with oocytes for 18 h IVF resulted in zygotes with defective fertilization.

3.4.3 Effect of DMSO in IVM followed by 18 vs 8 h IVF

This study was undertaken to investigate the effect of the MG132 solvent (0.03 M DMSO) during late maturation on post fertilization outcome. In previous studies, DMSO had a beneficial effect on blastocysts yield at 50 μM during bovine *in vitro* maturation, fertilization and culture (Tsuzuki et al., 1998, 2000). On the other hand, high (> 0.3 M) concentrations of DMSO have been shown to have a negative effect on GV breakdown in porcine oocytes during IVM (Li et al., 2016). The mechanism by which DMSO regulates cellular development has not yet been fully established. However, it is suggested that DMSO affects the production and stabilization of cytokines in different cells (Huang et al., 2016 and de Abreu Costa et al., 2017). This study showed that DMSO at 0.03 M during the last 6 h of maturation had no effect on post fertilization development of zygotes. However, as mentioned above, in comparison with conventional 18 h IVF, short incubation of oocytes (8 h IVF) reduced fertilization, but a greater proportion of zygotes developed to the blastocysts stage.

3.4.4 18 vs 8 h IVF post MG132 treatment in late maturation

To the best of our knowledge, this is the first study to compare two IVF duration periods following treatment of COCs with MG132 during late maturation on subsequent development of bovine embryos. The results of this experiment indicate that early denudation of COCs from cumulus cells post insemination eliminates the negative residual effect of MG132. Several studies reported a positive effect of MG132 during maturation on post fertilization outcome (You et al., 2012; 2012a; Ono et al., 2011 and Xiao et al., 2014). In agreement with You et al. (2012a), 10 μM MG132 during late maturation followed by 8 h sperm incubation with oocytes improved post fertilization development of zygotes.

As mentioned earlier (Chapter 2), MG132 reduces ATP production in oocytes and this reduction, if persistent, might lead to failure in ER Ca^{+} refill, resulting in post fertilization

defects. Although cumulus cells play an important role in the process of oocyte maturation and fertilization, the precise mechanism of their contribution still debatable (Tanghe et al., 2003 and Zhou et al., 2016). The negative residual effect of MG132 in conventional 18 h IVF, which is reversed with early denudation of zygotes, provides a new insight into the role cumulus cells play during fertilization. Also, gap junction Connexin levels are positively related with post fertilization outcomes, and insufficient flux of pyruvate through gap junctions from cumulus cells reduces ATP levels in oocytes and affects subsequent embryonic development (Wang et al., 2009). Moreover, gap junction protein degradation decreases post treatment with MG132 (Huang et al., 2010a and Nagyova, 2018). This might indicate that gap junction closure is delayed post treatment of bovine oocytes with MG132 during late maturation. On the other hand, ATP production by oocytes might be decreased following treatment with MG132 due to the reduction of ATP demand by the oocyte and infusion of ATP to cumulus cells to cover their demand. Moreover, as mentioned earlier, moderate reduction of intracellular ATP is a response to MG132 treatment inducing cell death in time dependant manner (Huang et al., 2010). However, it appears that maintaining certain levels of ATP during the first hours of fertilization is an essential requirement for development, and reduction of ATP post treatment of oocytes with MG132 could be rescued by short-term IVF in order to eliminate the residual effect of MG132 in cumulus cells. Therefore, short periods of gametes incubation, or early denudation of COCs post treatment with 10 µM MG132 during late maturation, seems to be a promising application for future research.

3.5 Conclusions

To the best of our knowledge, this is the first study to investigate the negative effects of MG132 administered during late oocyte maturation and to compare the effects of incubation period of gametes on subsequent post-fertilization development. Also, it is the first to investigate the effect of DMSO utilized during late maturation on the subsequent development of bovine oocytes. The results of this study revealed that 10 μM MG132 during late maturation followed by 18 h sperm incubation period reduces the development of zygotes. Although, short-term incubation of sperm with oocytes increases blastocysts of cleaved zygotes, this increase can be attributed to the reduction of cleaved zygotes at D2 of culture. Moreover, 10 μM MG132 followed by 8 h IVF has a beneficial effect on post fertilization outcome. Furthermore, the current study showed that DMSO at 0.03 M during late maturation had no effect on post fertilization development of zygotes. Further study for the association of ATP content, Ca^{+} oscillations and cyclin B during first few hours of fertilization following treatment of oocytes with MG132 during late maturation is required.

To investigate if the retardation of embryonic development that occurs during *in vitro* culture of bovine embryos beyond morulation could be enhanced by certain cytokines, the next chapter focuses on treatment of embryos at D5 of culture with a novel combination of Transforming Growth Factor B1 (TGFB1) and Colony Stimulating Factor 2 (CSF2).

Chapter 4: Effect of Granulocyte Macrophage Colony-Stimulating Factor and Transforming Growth Factor B1 on bovine embryo development

4.1 Introduction

Embryokines play an important role in regulating embryonic development during the pre- and post-implantation period. Moreover, they participate in the survival of transferred embryos. Although there are a few studies that reported no effect of CSF2 on embryonic development during bovine *in vitro* culture (Hickman et al., 2011), it was found to have a positive effect on embryo development in other studies (dMoras and Hanssen, 1997; Loureiro et al., 2009; Neira et al., 2010; Dobbs et al., 2013 and Sequeira et al., 2017). The improvement in embryonic development during the preimplantation period following CSF2 treatment is believed to be associated with upregulation of antiapoptotic genes (*NT5E, PRKAR2B* and *PGR*), and downregulation of pro-apoptotic genes (*MADD, RIPK3, NOD2* and *CREM*), together with altered expression of genes involved in cell cycle regulation and differentiation (Hansen et al., 2014; Loureiro et al., 2011), and antistressor genes (Wen et al., 2017).

On the other hand, TGFB pathway signalling is involved in many cellular processes including proliferation, differentiation, apoptosis and embryonic development (Li et al., 2012). Moreover, TGFB1 treatment from D4 of IVC increases the proportion of bovine zygotes reaching the blastocyst stage (Neira et al., 2010 and Moreno et al., 2015). TGFB1 signalling pathway is expressed in bovine embryos during the early preimplantation period (Kues et al., 2008 and Zuo et al., 2016). Although TGFB1 canonical signalling genes (*SMAD2*, *3* and *4*) are required for development of bovine embryos in early preimplantation (Lee et al., 2014), SMAD2, and 3 signals are diminished after the 8-cell stage during embryonic development *in vitro* (Zhang et al., 2015).

Little attention has been paid to the effect of combination of CSF2 and TGFB1 on embryonic development during the preimplantation period. However, Kanazaki et al.

(1995) refers to the importance of both cytokines in the growth and development of preimplantation embryos which is controlled by gonadal steroidal action. Also, TGFB1 in the presence of CSF2 promotes the growth of glial cells derived from dendriform cells *in vitro* (Xiao et al., 2002). Moreover, Suzuki et al. (2002) found that TGFB1 synergistically enhanced CSF2 augmented growth of umbilical cord blood CD34 (+), and FKH1 cells. However, in human myeloid leukaemia cell lines, TGFB1 inhibited PIK3 signalling stimulated by CSF2, which in turn reduced the proliferation of those cells (Montenegro et al., 2009).

Expression of CSF2 and TGFB1 in uterine tissues at different stages of pregnancy in the cat might indicate their roles in trophoblast invasion, vascularization, implantation and later in placentation (Agaoglu et al., 2016). With the foregoing discussion in mind, the following study was performed to investigate the effects of a novel combination of TGFB1 and CSF2 from D5 of culture on blastocyst yield. Also, to investigate if TGFB1 exhibits its effect during embryonic development in the cow through the *SMAD* signalling canonical pathway, the expression of TGFB1 related genes *(SMAD2, SMAD3)* were analysed.

4.2 Materials and methods

All chemicals used in this study were purchased from Sigma Aldrich chemical Co. unless otherwise stated. CSF2 is a bovine recombinant protein from Kingfisher Biotech Inc., USA (RP0871B) and was reconstituted with sterile phosphate–buffered saline containing 0.1% endotoxin free bovine serum albumin. TGFB1 is a human recombinant protein from Sigma Aldrich (H8541) and was dissolved in 4 mM HCl containing 0.1% endotoxin free bovine serum albumin. For immunostaining the reagents utilized were, Sex determining region Y-box 2 goat polyclonal (*SOX2*) (SC17320, Santa Cruz Bio-technologies, USA); Antigoat IgG Cyanine Dye3 (Cy3) (Jackson immune Research laboratories, Inc. USA, 705-165-003), Donkey serum (Sigma D9663) and mounting stain or Fluoroshield with 4′,

6-diamidino-2-phenylindole (DAPI) (Sigma, F6057).*In vitro* maturation, fertilization and culture were performed as previously described in Chapter 2 (Sections 2.2.2, 2.2.6 and 2.2.7). Embryos were allocated to treatments according to stage of development either at D2 of culture (46-48 h post insemination) or at D5 of IVC.

4.2.1 Effect of TGFB1 and CSF2 from D5 of IVC

Four replicated experiments with total of 417 graded COCs were utilized to investigate the effects of combinations TGFB1 and CSF2 at D5 of culture on blastocyst yields and quality. Embryos were allocated at D5 into one of three groups: (i) Control, (ii) Serum and (iii) TGFB1 + CSF2 groups. 10 ng ml^{-1} TGFB1 in combination with 50 ng ml^{-1} CSF2 were utilized at D5 of IVC. Fetal calf serum (10% FCS) was utilized as a positive Control. Blastocysts were harvested at D8 of IVC, counted, graded and immunofluorescently stained for cell number.

4.2.2 Effect of TGFB1 and TGFB1 + CSF2 from D5 of IVC

In order to investigate the effect of TGFB1 and or CSF2 at D5 of IVC, seven replicated experiments with a total of 777 graded COCs were utilized. Embryos were allocated at D5 into one of five groups: (i) Control, (ii) Serum, (iii) TGFB1, (iv) CSF2 and (v) TGFB1 + CSF2 (10 ng ml^{-1} TGFB1 and 50 ng ml^{-1} CSF2). Also, 10% FCS was utilized as a positive control. Blastocysts were harvested at D8 of IVC, counted, graded and immunofluorescently stained for cell number.

4.2.3 Effect of TGFB1 from D2-5 and CSF2 from D5-8 of IVC

In seven replicated experiments, a total of 839 graded COCs were utilized to investigate the effects of 10 ng ml^{-1} TGFB1 between D2-5 and between D5-8 either separately or in combination with 50 ng ml^{-1} CSF2. Embryos were allocated at D2 into one of four groups: (i) Control, (ii) TGFB1-D2-5, (iii) CSF2-D5, and (iv) TGFB1-D2-5 + CSF2-D5 and

reallocated at D5 between relevant groups. Blastocysts were harvested at D8 of IVC, counted, graded and immunofluorescently stained for total cell number and inner cell mass.

4.2.4 Immunofluorescent staining

To investigate the effect of treatment with cytokines on total cell count and Inner cell mass for embryo quality, blastocysts were collected at D8 of IVC and stained by immunofluorescence. *SOX2* was used as primary antibody and Cy3 as secondary antibody for the staining of inner cell mass (ICM) (Avilion et al., 2003 and reviewed by Zhang and Cui, 2014). Also, mounting stain or fluoroshield with DAPI was used for the staining of the trophoblast. Briefly, zona pellucida were removed from harvested blastocysts at D8 of IVC utilizing Pronase 0.5 % w/v for around 1 min. Then, blastocysts were washed with warm PBS-PVP (0.1% v/v) and transferred to 4% paraformaldehyde for 15 min for fixation followed by washing with PBS-PVP, and either used directly for staining or stored in a moist condition at 4°C for up to one week. In the day of staining, zona-free blastocysts were washed three times for 5 min each in 1% BSA-PBS before being transferred to 0.1% triton-X100 in PBS for 15 min at room temperature for permeabilization. Then blastocysts were washed twice in 1% BSA-PBS for 5 min each and transferred into blocking solution (containing 82% of 5% BSA-PBS, 9% Donkey serum and 9% 0.3M Glycine) for 1 h at room temperature. After that, blastocysts were transferred to a 96 mini-well plate that loaded with primary antibody (*SOX2*) and kept overnight in a moist condition at 4°C. Then, blastocysts were washed four times for 10 min each in 1% BSA-PBS and transferred into 96 mini-well plate loaded with secondary antibody (Cy3) and kept for 1 h at room temperature. After that, blastocysts were washed four times for 10 min each in 1% BSA-PBS and mounted with minimal volume of medium into SLS immune-fluorescent 8 mm 5-well slides loaded with a few microliters of fluoroshield-DAPI. Finally, mounted blastocysts with media were covered with a coverslip

and sealed with nail varnish and visualized for inner cell mass and trophoblast using an epifluorescent microscope (Leica, DM4000B; Germany).

4.2.5 Effect of TGFB1 from D2-7 on *SMAD* expression

TGFB1 was utilized at 10 ng ml^{-1} on D2 to D7 of IVC in four replicated experiments to investigate its effect on *SMAD2* and *SMAD3* transcript expression. Also, in order to determine the effect of dose on expression of TGFB1 canonical pathway related genes, three replicated experiments utilizing 50 ng ml^{-1} TGFB1 were performed. Media was changed at D5 of IVC. Embryos at morula and blastocysts stages were collected on D7 of IVC in 2 µl PBS-PVP 0.1%, followed by snap freezing and kept in -80 until evaluation.

4.2.6 RNA extraction

mRNA was extracted utilizing dyna-beads mRNA direct purification kit (Invitrogen-Cat.no. 61011) according to the manufacturer's instructions. Briefly, embryos were pooled to 7, 8 and 10 embryos and lysed in 150 µl lysis/binding buffer. 40 µl of dynabeads® oligo $(dT)_{25}$ were added to the lysis mixture and left for 10 min at room temperature with rotation. The mixture was then subjected to pulse centrifugation and tubes then exposed to magnet for a few seconds or until the beads formed a pellet. After that, the supernatant was removed using gel loading tips and the beads were treated three times with the following repeated steps: (i) washing with 100 µl buffer-A, (ii) pulse centrifugation and (iii) magnetic exposure. The beads were then treated with two repeated steps of wash with 100 µl buffer-B, followed by pulse centrifugation and magnetic exposure with removing the supernatant. After that, 10 µl RNase free water were added to the beads, resuspended and incubated at 65ºC for 2 min. Finally, the tubes were transferred to ice with magnetic exposure (solution chilled to prevent mRNA binding to the beads), and the supernatant that contained the mRNA was transferred to a new tube and stored at -80 °C for cDNA synthesis. (**Figure 4.1**).

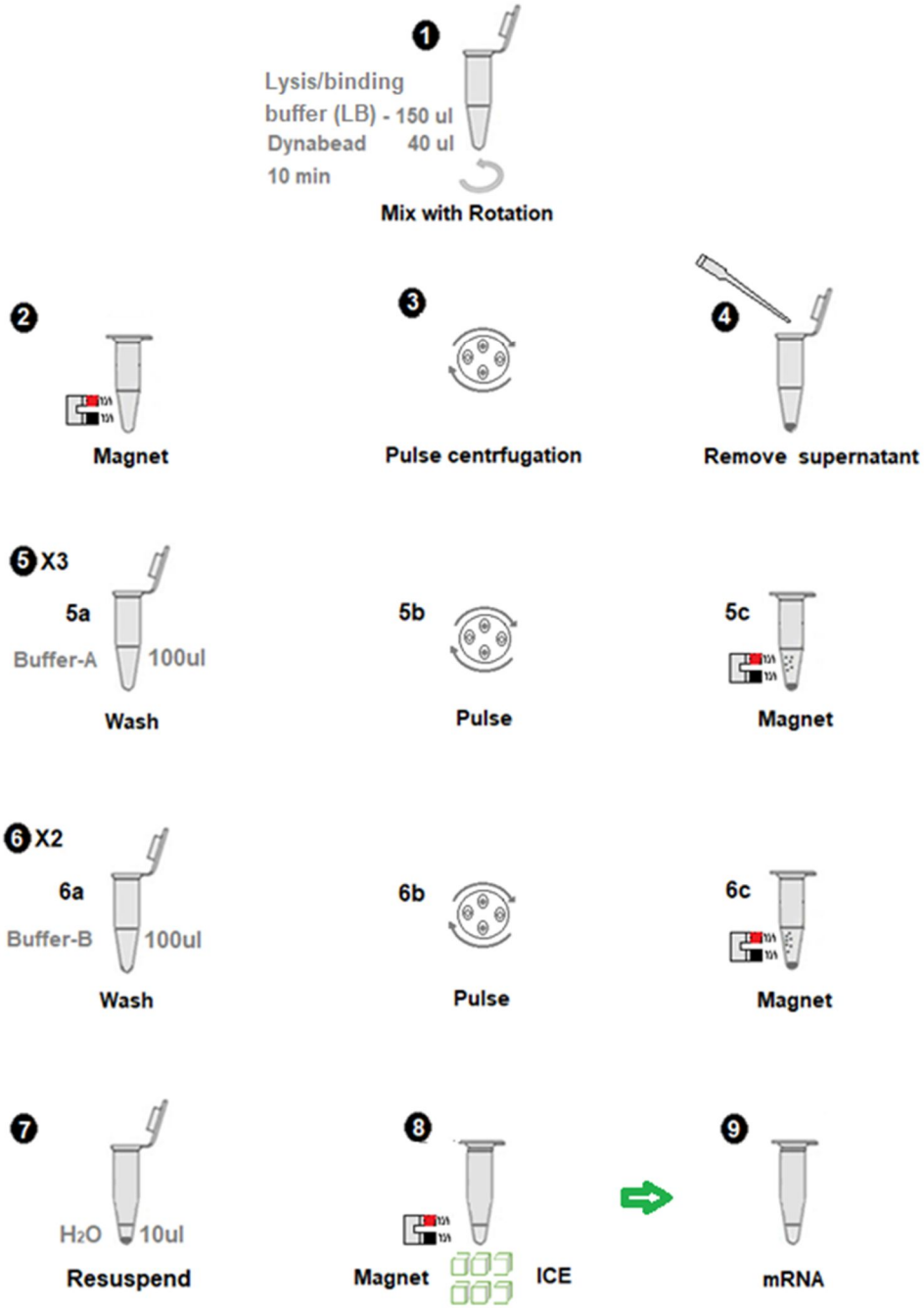

Figure 4.1. Illustrative diagram for the steps of RNA extraction utilizing dyna-beads mRNA direct purification kit.

4.2.6.1 Reverse transcription and cDNA synthesis

The complementary DNA were prepared using QuantiTect Reverse transcription kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instruction. Briefly, Quantiscript RT buffer was mixed with RT primer mix at room temperature to form gDNA wipeout buffer (DNase). Then 10 µl of dynabeads mRNA were added to 2 µl DNase and 2 µl RNase free water and mixed by pipetting, pulse centrifugation and incubation at 42 °C for 2 min. After that, 13 µl of DNase treated RNA was used for PCR reaction (+RT) and 1 µl used as a negative control (-RT). The total volume of each PCR reaction (+RT) was 20 µl, containing 1 µl Quantiscript reverse transcriptase; 4 µl Quantiscript RT buffer (5x); 1 µl RT Primer mix (from the kit that contains random primers and oligo dT); 1 µl diluted gDNA Wipeout buffer (7x buffer1:6 RNase free water) and 13 µl DNase treated RNA. The PCR reaction was mixed by flicking the tube with pulse centrifugation. Then the reaction tubes were incubated at 42°C for 30 min, and 95°C for 3 min to inactivate the enzyme. For negative control (-RT), 1 µl RNase free water was added to 1 µl DNase treated RNA and incubated at 95°C for 3 min. The resultant cDNA was used as a template for amplification. All products were stored at -20°C for quantitative real time polymerase chain reaction (qRT PCR).

4.2.6.2 Quantitative real time polymerase chain reaction (qRT PCR)

The relative quantification of *SMAD2* and *SMAD3* was analysed by qRT-PCR utilizing SYBR Green fluorescent dye. Forward and reverse primers for *SMAD2*, and *SMAD3* were adopted from Zhang et al. (2015) and primers for *RPS18* were designed using Primer Express software (**Table 4.1**).

Table 4.1 Primers and sequences utilized in rt-PCR for *SMAD2*, and *SMAD3*

Genes	Accession no.	sequence (5'->3')
SMAD2	NM_001046218.1	F:5'-GAGGTGGCGTTTCTGGGATA-3' R: 5'-TGCTCTGAAATTTGGGGACTGA-3'
SMAD3	NM_001205805.1	F: 5'-GGAGGTAGAACTGGGGTCTCT-3' R: 5'-GCTGGAAAAAGGGCGAGCA-3'
RPS18	NM_001033614.2	F: 5'-GGATCTTGTATTGGCGTGGATT-3' R: 5'-CACCGAGGATGAGGTGGAA-3'

Primers were supplied by Eurofins Genomics (GmbH, Anzinger Str. 7A, 85560, Ebersberg, Germany). The primers were diluted with RNase free water. Quantification of the related genes was performed in a total volume 10 µl, including 5 µl LightCycler 480 SYBR Green Master I master mix, 0.2 µl (10 mM) of each forward and reverse primers, 2.8 µl PCR water and 2 µl diluted cDNA (equivalent cDNA to 0.5 embryo / reaction and diluted to 2 µl with PCR water). All samples and controls were subjected to initial denaturation at 95 °C for 10 min before running PCR. Amplification was performed in thermal Light Cycler for 50 cycle and in three temperature steps: (i) denaturation at 95°C for 5s, (ii) annealing at 60 °C for 25s, and (iii) extension at 72 °C for 15s. CP values were analysed by second derivative maximum method and utilized in the calculation of relative expression of *SMAD2* and *SMAD3* to the reference gene (RPS18) and based on $R=2^{\wedge-(\Delta\Delta CP)}$ for Control and samples (Pffafil et al., 2012). The resultant values were then normalized by dividing them on ΔΔCP value (Target-reference) of the Control, so that the control normalized to 1.

Melting curve analysis was performed to identify non-specific products in the reaction. The data revealed specificity of the test for the relative quantification of selected genes which were presented by amplification curves, melting peak and melting curves for *RPS18*, *SMAD2* and *SMAD3* (**Figure 4.2**).

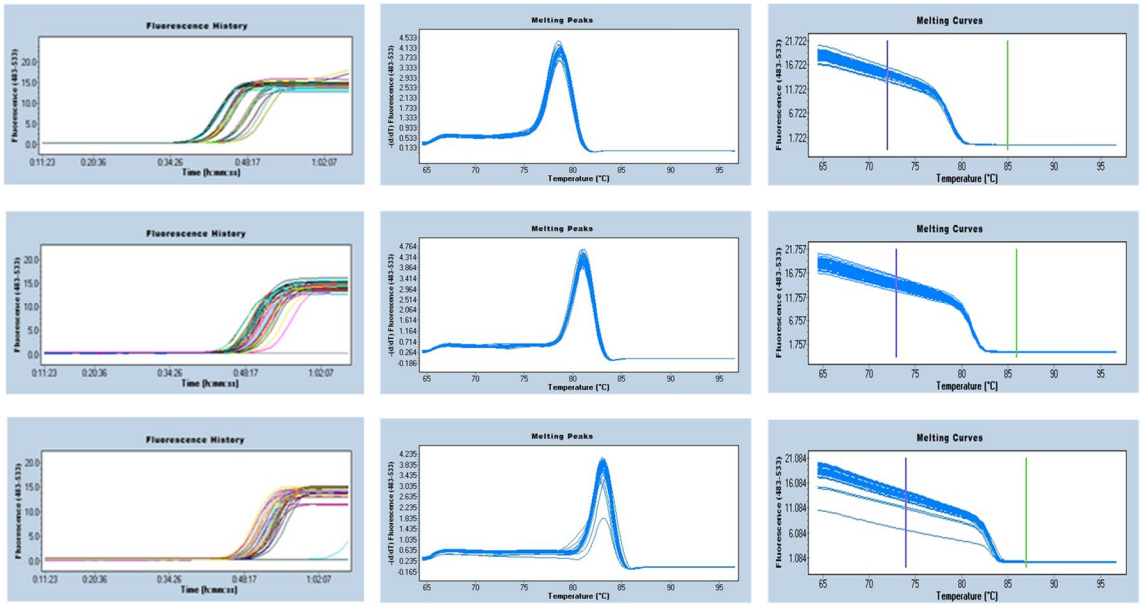

Figure 4.2. Amplification curve, melting peak and melting curves for *RPS18* (Top), *SMAD2* (Middle) and *SMAD3* (Bottom).

4.2.7 Statistical analysis

Statistical analyses were performed utilizing GenStat statistical package (17th Edition, VSN International, 2011). All proportion data were analysed using generalized linear regression models that assumed binomial errors and used logit-link functions. Analyses of cell number (inner cell mass and trophectoderm) used generalized linear regression models but assumed Poison errors and used log-link functions. Transcript expression was analysed by analysis of variance (ANOVA). Residual plots confirmed that data were normally distributed and homogeneous. Data are presented as predicted means and standard error of these means (s.e.m). In each case, differences between individual means were established using Least Significant Differences (LSD) derived from residual errors of the statistical models. Differences were considered significant at $P<0.05$.

4.3 Results

4.3.1 Effect of TGFB1 and CSF2 from D5 of IVC

The proportion of cleaved zygotes and the ≥ 4 cell embryos from inseminated oocytes, and from cleaved zygotes are presented in **Figure 4.3A**. The proportion of 2 cell, 3 cell, 4 cell and > 4 cell embryos from cleaved zygotes at D2 of in vitro culture is presented in **Figure 4.3B**.

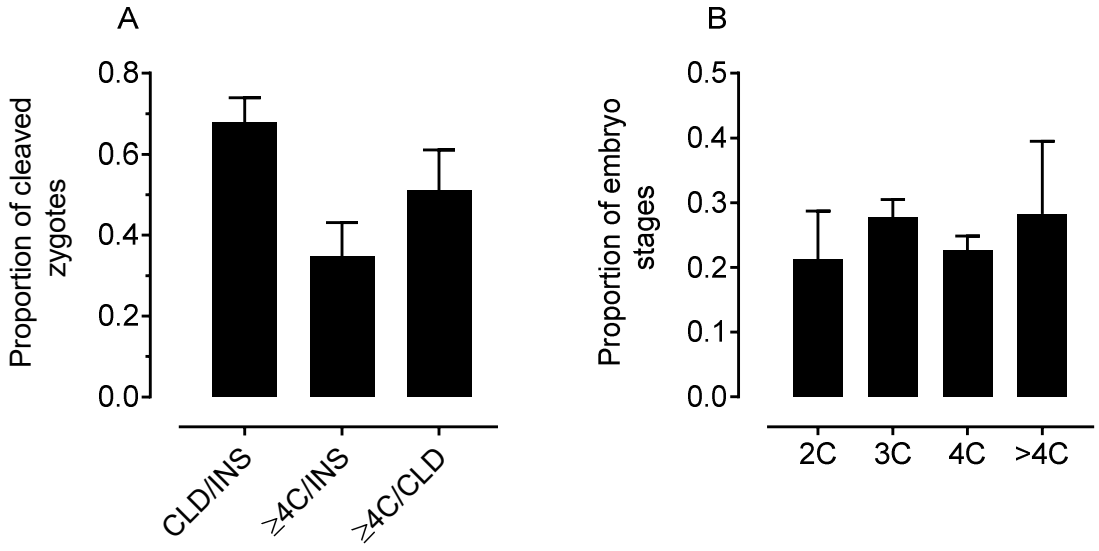

Figure 4.3. Proportion of cleaved zygotes (CLD) from inseminated oocytes (INS) and ≥4 cell embryos from inseminated oocytes and cleaved zygotes (A), and embryo developmental stages (B) at D2 of *in vitro* culture. Experiment replicated four times.

The combination of 10 ng ml^{-1} TGFB1 and 50 ng ml^{-1} CSF2 from D5 of culture reduced (P=0.016) the proportion of blastocysts from cleaved zygotes at D8 of IVC. Also, adding serum to media from D5 increased blastocyst proportion (P=0.03) in comparison with the growth factor combination group (**Figure 4.4A**). On the other hand, treatment of embryos from D5 with TGFB1 plus CSF2 had no effect on total cell number in early and late categories of 39 analysed blastocysts (**Figure 4.4B**).

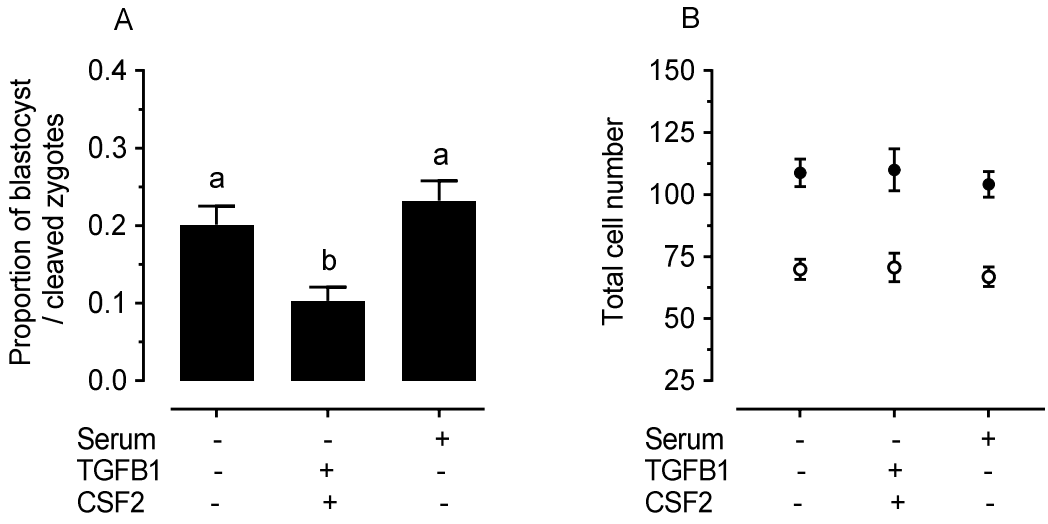

Figure 4.4. Proportion of blastocyst from cleaved zygotes at D8 of IVC (A), and total cell number (B) in the ○ early and ● late blastocysts from embryos treated with TGFB1 and CSF2 from D5 of IVC. Means with different superscripts are different at P<0.05. Experiment replicated four times (n=39).

4.3.2 Effect of TGFB1 or TGFB1 + CSF2 from D5 of IVC

The proportion of cleaved zygotes from inseminated oocytes, ≥ 4 cell embryos from inseminated oocytes and cleaved zygotes at D2 are presented in **Figure 4.5A**. Also, the proportion 2 cell, 3 cell, 4 cell and > 4 cell of cleaved zygotes at D2 are presented in **Figure 4.5B**.

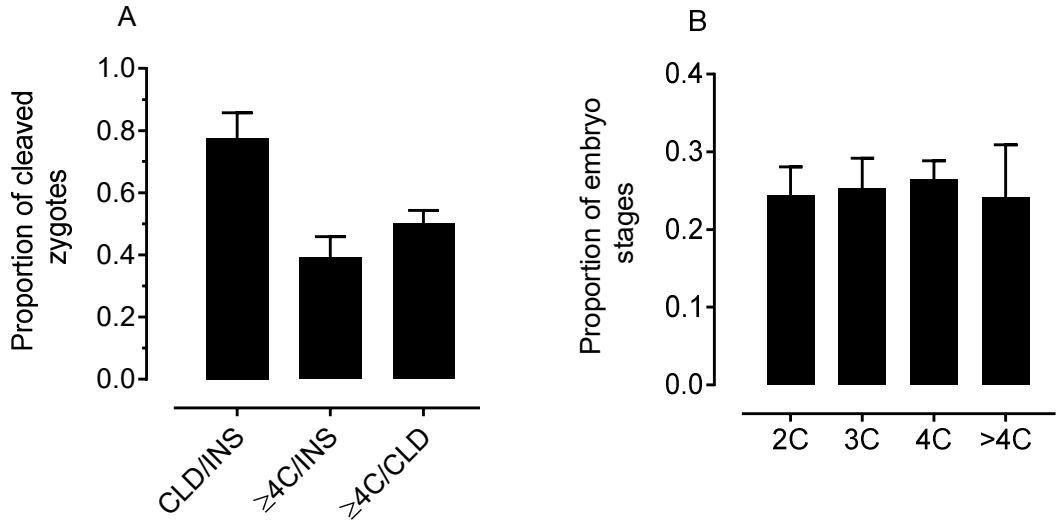

Figure 4.5. Proportion of cleaved zygotes (CLD) from inseminated oocytes (INS) and ≥4 cell embryos from inseminated oocytes, and cleaved zygotes (A), and embryo developmental stages (B) at D2 of *in vitro* culture. Experiment replicated seven times.

Although serum increased (P=0.006) the proportion of zygotes reaching the blastocyst stage at D8 of IVC, the addition of TGFB1 in combination with CSF2 from D5 of culture eliminated (P=0.025) the positive effect of CSF2 on this proportion (**Figure 4.6**). TGFB1 on its own had no effect on the proportion embryos reaching the blastocysts stage by D8.

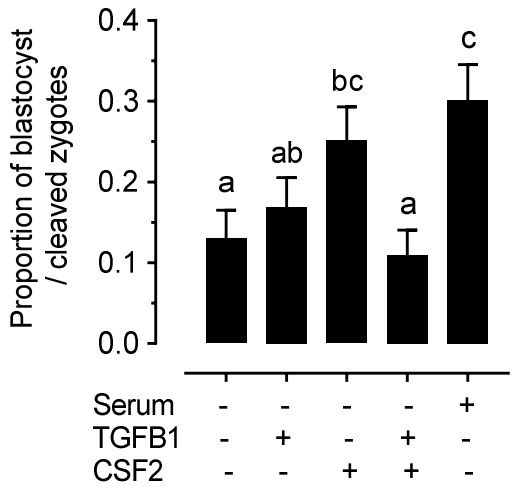

Figure 4.6. Proportion of blastocysts at D8 of IVC from cleaved zygotes post treatment of embryos with TGFB1 and CSF2 from D5 of culture. Means with different superscripts are different at P<0.05. Experiment replicated seven times.

On the other hand, and in total 101 analysed blastocysts, the total cell number was not different between groups for early or late blastocysts post treatment of embryos with TGFB1 or TGFB1 + CSF2 from D5 of IVC (**Figure 4.7A**). The same was true also for cell number within the inner cell mass in the late blastocysts (**Figure 4.7B**).

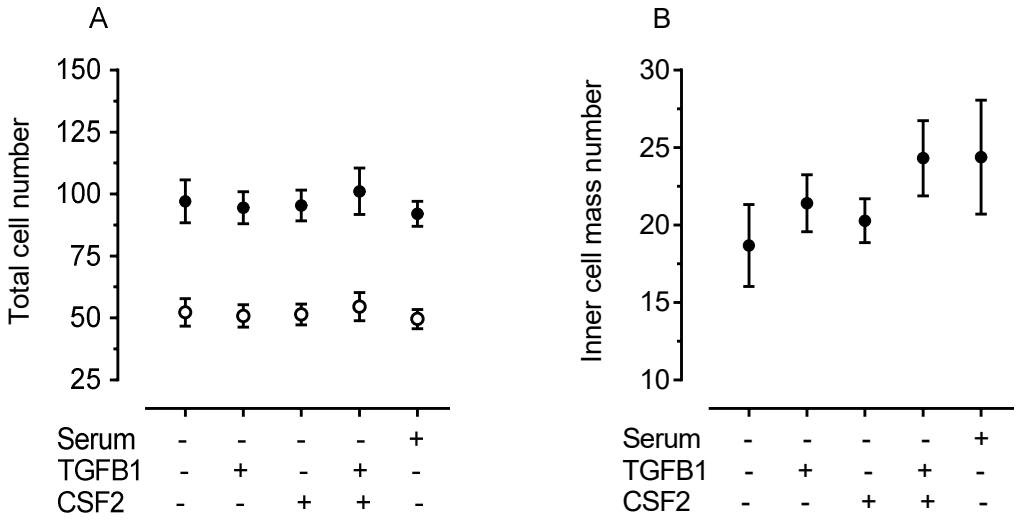

Figure 4.7. Total cell number (A) and Inner cell mass (B) for blastocysts at D8 of IVC post treatment of embryos with TGFB1 and CSF2 in D5 of culture. Experiment replicated seven times (n=101).

4.3.3 Effect of TGFB1 from D2-5 and CSF2 from D5-8 of IVC

The proportion of cleaved zygotes, ≥ 4 cell embryos from inseminated oocytes and cleaved zygotes at D2 of culture are presented in **Figure 4.8A**. Also, the proportion 2-cell, 3- cell, 4- cell and > 4- cell embryos for total replicates at D2 of culture are presented in **Figure 4.8B**.

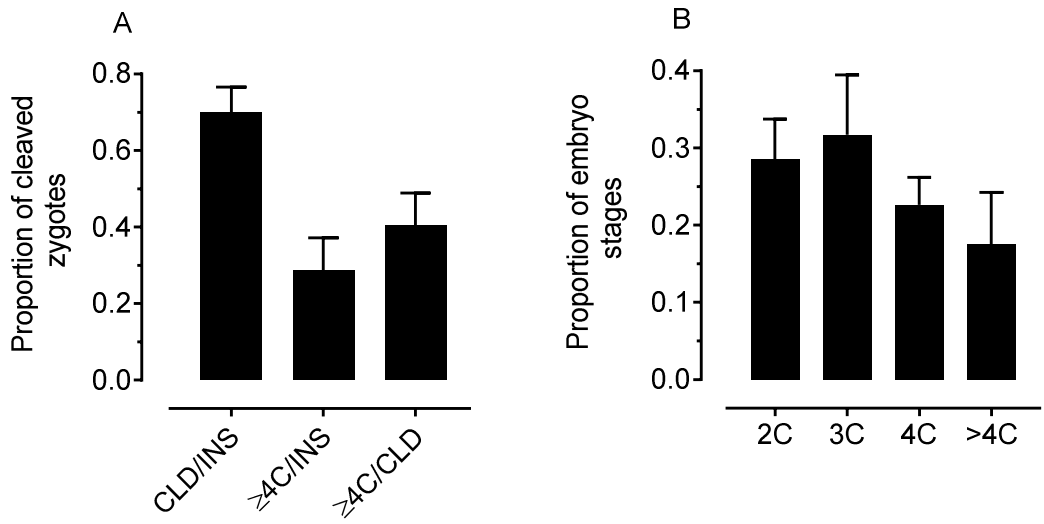

Figure 4.8. Proportion of cleaved zygotes of inseminated oocytes and ≥4 cell embryos from of inseminated oocytes, and cleaved zygotes (A), and embryo developmental stages (B) at D2 of *in vitro* culture. Experiment replicated seven times.

CSF2 added from D5 of culture increased (P=0.004) the proportion of blastocyst at D8 of IVC from cleaved zygotes. However, the addition of TGFB1 from D2-5 individually and from D5-8 in combination with CSF2 had no effect on the proportion of blastocysts at D8 of *in vitro* culture (**Figure 4.9**).

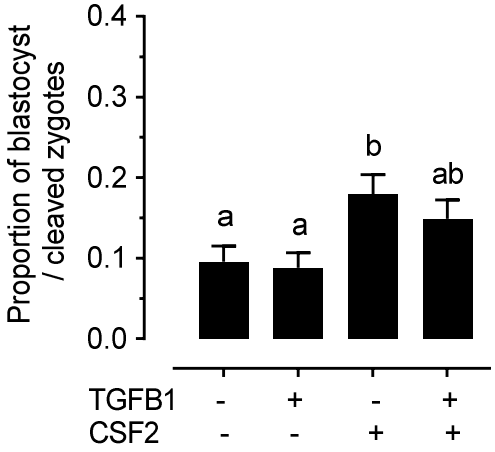

Figure 4.9. Proportion of blastocysts from cleaved zygotes at D8 post treatment of embryos with TGFB1 on D2-5 and CSF2 D5-8. Means with different superscripts are different at P<0.05. Experiment replicated seven times.

On the other hand, and in total 64 analysed blastocysts, total cell number was not different between groups for early and late blastocysts (**Figure 4.10A**). However, CSF2 increased cell number within the inner cell mass for both early and late blastocysts (**Figure 4.10B**) as assessed by immunostaining (**Figure 4.11**).

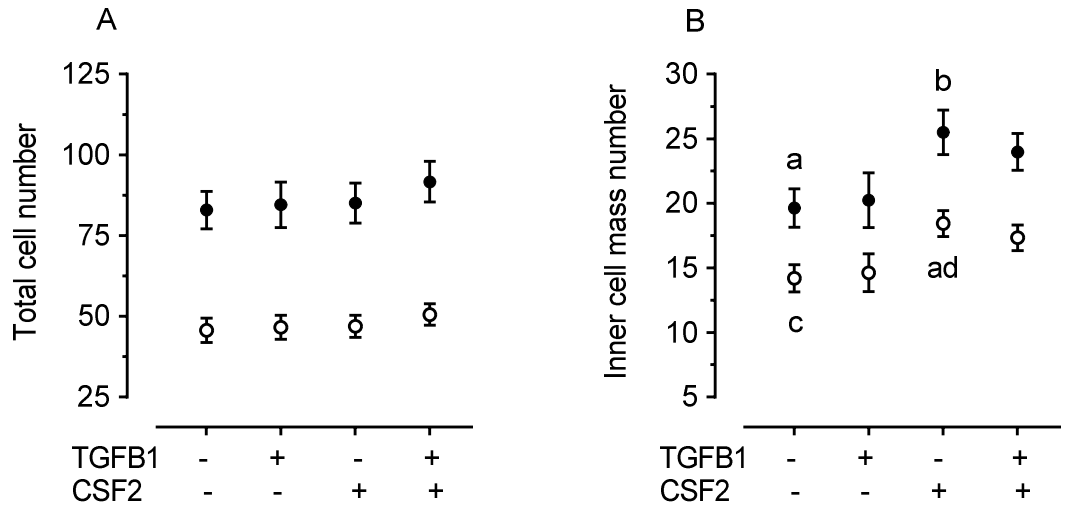

Figure 4.10. Total cell number (A), and inner cell mass (B) in early and late blastocysts at D8 of culture and post treatment of embryos with TGFB1 from D2-5 and CSF2 from D5-8 of IVC. Experiment replicated seven times (n=64).

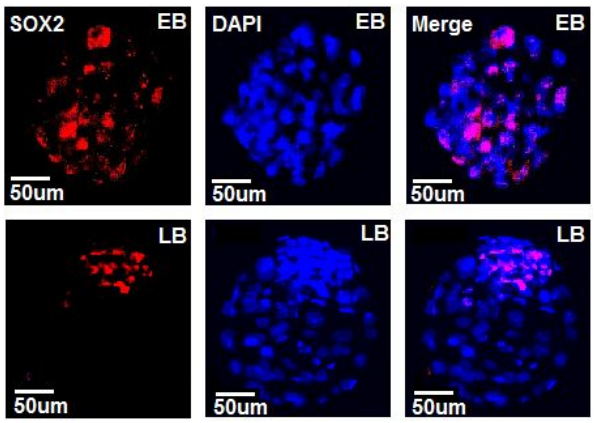

Figure 4.11. Immunostaining of bovine blastocysts by SOX2 at D8 of *in vitro* culture in early (Top) and late (bottom) blastocysts post treatment of embryos with TGFB1 from D2-5 and CSF2 at D5-8.

4.3.4 Effect of TGFB1 from D2-7 on *SMAD* expression

Treatment with TGFB1 between D2 to D7 of IVC had no effect on expression of *SMAD2* and *SMAD3* in bovine embryos. Moreover, with respect to the dose of TGFB1, 50 ng ml^{-1} was similar to 10 ng ml^{-1} and had no effect on the expression of *SMAD2* or *SMAD3* (**Figure 4.12**).

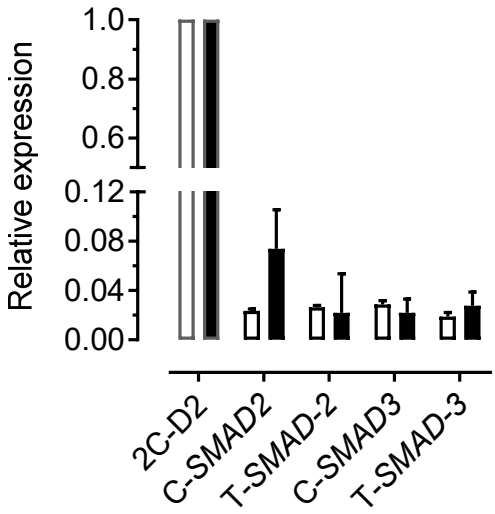

Figure 4.12. Relative expression of *SMAD2* and *SMAD3 at* D7 for morula and blastocyst stage post treatment of embryos from D2-7 either with 10 ng ml^{-1} (white bar) or 50 ng ml-1 (black bar) TGFB1. Data are for 2 cell embryos at D2 (2C-D2), Control *SMAD2* (C-*SMAD2*), Treated *SMAD2* (T-*SMAD2*), Control *SMAD3* (C-*SMAD3*) and Treated *SMAD3* (T-*SMAD3*). Experiment replicated seven times.

4.4 Discussion

The current study was performed to investigate the effect of a novel combination of TGFB1 and CSF2 from D5 on blastocysts yield and quality. Also, to investigate the effect of TGFB1 during culture on *SMAD2* and *SMAD3* expression in embryos at D7 of IVC. The results of this study revealed that TGFB1 reduced blastocyst yield when combined with CSF2 but had no effect on cell number. CSF2 on its own increases blastocyst yield and cell number within the inner cell mass. Finally, the current study showed that TGFB1 during culture, at 10-50 ng ml $^{-1}$, had no effect on *SMAD2* and *SMAD3* expression in embryos at D7 of IVC.

4.4.1 Effect of TGFB1 and CSF2 from D5 of IVC

Unexpectedly, the results of the current experiment indicated that the novel combination of TGFB1 and CSF2 from D5 reduced blastocyst development. However, this

combination had no effect on total cell number of blastocysts at D8 of *in vitro* culture. The mechanism by which the combination of TGFB1 and CSF2 from D5 of culture reduces the development of bovine embryos is unknown. However, in tumor cells, the *SMAD* signaling pathway (i.e. the TGFB1 canonical pathway) inhibits cell proliferation and promotes apoptosis (Sherbet, 2011). Moreover, it has been shown that TGFB1 has cytostatic activity in different human epithelial cells through stimulation of the *SMAD* signaling pathway (mainly *SMAD3*) that might stimulate cyclin dependent kinase inhibitors (p15 and p21) in the case of proliferation (Kim et al. 2005). In contrast, TGFB1 inhibits PIK3 which is stimulated by CSF2 and leads to suppression of proliferation of human leukemic cells (Montenegro et al. 2009). Based on these findings, TGFB1 might reduce the development of embryos induced by CSF2 either through stimulation of *SMAD* signaling pathway or by interfering with the action of CSF2 through the non-canonical pathway.

4.4.2 Effect of TGFB1 and TGFFB1 + CSF2 from D5 of IVC

This study was undertaken to investigate the effect of TGFB1 individually or in combination with CSF2 from D5 of culture on blastocyst yield and quality. In accord with previous studies (dMoras and Hanssen, 1997; Loureiro et al., 2009; Neira et al., 2010; Dobbs et al., 2013 and Siqueira et al., 2017), CSF2 enhanced the development of embryos to the blastocyst stage. However, the combination of TGFB1 and CSF2 had no effect on the development of embryos to the blastocyst stage in the current study. It has been demonstrated that CSF2 increases blastocyst yield only when the level of embryonic development in culture is low (Dobbs et al. 2013). Our results showed that CSF2 administered from D5 enhances the number of cells in the inner cell mass in resultant bovine blastocysts at D8 of *in vitro* culture. It has previously been demonstrated that CSF2 increases the inner cell mass of bovine blastocysts at D8 of *in vitro* culture (Loureiro et al., 2009). On the other hand, TGFB1 at the current concentration (10 ng/ml) had no effect on development of embryos to the blastocyst stage. Also, TGFB1 had no

effect on total cell number or inner cell mass at D8 of *in vitro* culture. It has been shown that TGFB1 at 50 ng ml^{-1} has a beneficial effect on the development of bovine embryos to the blastocyst stage (Neira et al., 2010 and Moreno et al., 2015). However, TGFB1 at 2 ng ml^{-1} had no effect on the development of bovine embryos and cell number of resultant blastocysts (Keefer, 1992). It seems that TGFB1 at the current concentration had no effect on the proliferation signals in embryos. Therefore, further investigations into different concentrations of TGFB1 during preimplantation period of bovine embryos are required.

In terms of the combination of TGFB1 with CSF2 from D5 of *in vitro* culture, TGFB1 suppresses the stimulatory effect of CSF2 on the development of embryos to the blastocyst stage but had no effect on cell number of the resultant blastocysts. There are no studies that have assessed the effects of the combination TGFB1 and CSF2 on embryo development. However, it has been shown that TGFB1 inhibits the stimulatory effect of CSF2 through suppression of PIK3 in human myeloid leukemic cells (Montenegro et al., 2009). On the other hand, and in accord with the earlier stated studies in section 4.5.2, CSF2 individually increases the development of embryos to blastocysts stage. Also, as mentioned earlier, CSF2 has a stimulatory effect on proliferation of bovine embryos and TGFB1 suppresses this effect either by stimulation of canonical *SMAD* signalling pathway or through interfering with the action of CSF through the non-canonical signalling pathway.

4.4.3 Effect of TGFB1 from D2-5 and CSF2 from D5-8 of IVC

As per our previous results in Sections 4.4.1 and 4.4.2, TGFB1 reduces the stimulatory effect of CSF2 in relation to embryonic development when combined from D5 of *in vitro* culture. This emphasises the importance of TGFB1 signalling pathway in early preimplantation (Kues et al., 2008; Zhang et al., 2015 and Zuo et al., 2016). This study was performed to investigate the effect of utilizing TGFB1 from D2-5 and CSF2 from D5-

8 of *in vitro* culture on the development of bovine embryos and blastocysts quality. TGFB1 from D2-5 of culture had no effect on the development of bovine embryos to blastocysts stage and the cell number in the resultant blastocysts at D8 of culture. Recently, it was shown that treatment of bovine embryos for the first 48 h of culture with either 50 or 100 ng ml^{-1} TGFB1 had no effect on the development of zygotes to the blastocyst stage (Barrera et al., 2018). In accordance with the previous experiment (Section 4.5.2), CSF2 increases the development of embryos to the blastocyst stage. Moreover, in agreement with Loureiro et al. (2009), CSF2 administered from D5 of culture either individually or when preceded with TGFB1 from D2-5 of culture increases inner cell mass of the resultant blastocysts.

4.4.4 Effect of TGFB1 from D2-7 on *SMAD* expression

To investigate if TGFB1 affects proliferation throughout the canonical *SMAD* signalling pathway during the development of bovine embryos cultured *in vitro*, the current study assessed the effect of TGFB1 from D2-7 on the expression of *SMAD2* and *SMAD3* at D7 of *in vitro* culture. It also investigated the effect of increasing TGFB1 dose on the expression of these TGFB1 signalling genes. The results of the current study revealed, for the first time, that treatment of bovine embryos with 10 ng ml^{-1} TGFB1 between D2-7 had no effect on the expression of *SMAD2* and *SMAD3*. Moreover, increasing TGFB1 dose to 50 ng ml^{-1} had no effect on these two TGFB1 signalling related genes. It has been shown that expression of *SMAD2* and *SMAD3* is highly reduced beyond 8-cell stage for bovine embryos cultured *in vitro* (Zhang et al., 2015). It seems that TGFB1 interferes with the non-canonical pathway to eliminate the stimulatory effect of CSF2 on development of bovine embryos cultured *in vitro*. However, this was not confirmed in the current study and so further investigation in regard to the precise signalling pathway that explains this interference is required.

4.5 Conclusions

This is the first detailed study to investigate the effects of TGFB1 in combination with CSF2 from D5 of *in vitro* culture on blastocyst yield and quality in bovine. Also, it is the first to investigate the possible role of TGFB1 signalling canonical pathway related genes (*SMAD2* and *SMAD3*) in the development of bovine embryos. These series of experiments revealed that TGFB1 eliminates the stimulatory effect of CSF2 when both are utilized from D5 of *in vitro* culture. Also, that CSF2 when utilized alone from D5 of culture has a beneficial effect on blastocyst yield and quality in terms of the number of cells within the inner cell mass. TGFB1 at 10 ng ml^{-1} from D5 of culture had no effect on the development of bovine embryos cultured *in vitro*. Moreover, TGFB1 from D2-5 had no effect on embryonic development in the current concentration (10 ng ml^{-1}).

On the other hand, TGFB1 had no effect on *SMAD2* and *SMAD3* expression when added between D2 to 7 of culture. The latter observation suggests that the mode of action of TGFB1 in suppression of CSF2 post D5 of culture is through a non-canonical signalling pathway. Further investigation in regard to the signalling pathway that inhibited the combination of TGFB1 and CSF2 from D5 of *in vitro* culture and the effect of using different concentrations of TGFB1 during different time points of embryonic development are required.

As previously shown in Chapters 2 and 3, MG132 during late maturation followed by 8 h incubation of gametes for fertilization increases number of bovine zygotes reaching blastocyst stage. Also, as shown earlier in this chapter, CSF2 administered from D5 of culture has a beneficial effect on the development of bovine embryos during preimplantation. In order to investigate if embryonic development could be further enhanced, the next chapter focused on the effect of a combination MG132 during maturation, followed by 8 h IVF with CSF2 added at D5, on blastocyst yield and sex ratio of bovine embryos.

Chapter 5: Effect of combined treatment of MG132 and CSF2 on development and sex ratio of bovine embryos

5.1 Introduction

Developmental competence of bovine embryos during the preimplantation period is influenced by numerous factors including oocyte maturation status, duration of IVF and composition of culture media. Also, those factors can influence sex ratio of embryos cultured *in vitro*. It was reported previously that the optimal period of oocyte-sperm incubation is 18-24 h (Rehman et al., 1994 and Nedambale et al., 2006). However, Ward et al. (2002) showed that 10 h insemination period is sufficient for optimal fertilization, but sperm concentration and the difference between sires in sperm penetration are other factors that affect blastocysts yield. Although King et al. (1991) demonstrated that the male: female ratio is not different from 1:1 in bovine embryos produced *in vivo* and *in vitro*, Iwata,H. (2012), proposed factors affecting sex ratio during preimplantation period of bovine embryos that includes sperm-oocyte status and timing of insemination. Also, culture media composition might play an important role in skewing the sex ratio of bovine blastocysts (Gutierrez-Adan et al., 2001 and Siqueira and Hansen, 2016).

Prolonged *in vitro* oocyte maturation skewed sex ratio towards male embryos in cattle (Dominko and First, 1996 and Agung et al., 2006). On the other hand, short periods of gamete incubation (of around 6 h) reduces fertilization in the cow but produces high yields of blastocysts from cleaved zygotes with an increased proportion of male blastocysts (Kochhar et al., 2003). CSF2 regulates a variety of genes related to the development of both cell lineages in embryos, as well as the elongation of bovine embryos (Dobbs et al., 2014; Hansen et al., 2014 and Ozawa et al., 2016). Moreover, Siqueira and Hansen, (2016) showed that following the use of X or Y sorted semen, CSF2 increases the development of cleaved zygotes to blastocysts, but in favour of female rather than male embryos. Furthermore, the sex of embryos exhibit differences

in DNA methylation during preimplantation development and sex also affects the development of bovine embryos cultured *in vitro* (Dobbs et al., 2013a).

As previously reported, MG132 during late IVM had a beneficial effect on post-fertilization development when followed by a short (8 h) period of insemination (Chapter 3). On the other hand, CSF2 alone at D5 of IVC enhanced the development of embryos to the blastocyst stage and increased cell number within the inner cell mass of blastocysts (Chapter 4). Based on these results, the following series of experiments tested the hypothesis that treatment of bovine oocytes with MG132 during late maturation followed by a short period incubation of gametes during fertilization, with CSF2 administered from D5 of *in vitro* culture increases blastocyst yields and skews the sex ratio of bovine blastocysts in favour of female. Therefore, this study was carried out to investigate the effect of MG132 during late maturation followed by either 8 h or 18 h IVF period, and CSF2 added at D5 of IVC on blastocyst yield and sex ratio of bovine blastocysts on D8 of culture.

5.2 Materials and methods

Except for embryo sexing, all chemicals and procedures utilized during IVM, IVF and IVC were described previously in Chapters 2, 3 and 4. Briefly, after aspiration, Grade 1 and 2 COCs were selected and allocated to groups matured for 16 h. 10 µM MG132 was added during the final 6 h of IVM. Post 22 h maturation, COCs were partially denuded and transferred to fertilization media for either 8 or 18 h incubation. Zygotes were then fully denuded and cultured in microdroplets covered with oil. On D2 (~46-48 h of insemination), zygotes were monitored for cleavage and cleaved zygotes transferred to new culture media. Embryos were observed again on D8 of *in vitro* culture and analysed for the determination of embryo sex.

5.2.1 Effect of MG132 and CSF2 (8 h vs 18 h IVF) on proportion of blastocyst

A total of 411 graded COCs were utilized in four replicated experiments to investigate the effect of treatment with MG132 plus CSF2, the latter following either 8 or 18 h IVF, on the proportion of blastocyst and embryo sex ratio at D8 of IVC. COCs were allocated into one of five groups: (i) Control, 18 h; (ii) CSF2, 18 h; (iii) Control, 8 h; (iv) MG132, 8 h; and (v) MG132, 8 h plus CSF2. Cleaved zygotes were allocated at D2 and re-allocated at D5 of *in vitro* culture according to the stage of development between relevant groups without affecting the total number of embryos per group in order to minimize variation during subsequent embryo development. Therefore, Control, 18 h groups were allocated with CSF2 groups. Also, MG132, 8 h groups were allocated with MG132, 8 h plus CSF2 groups. At D8 of IVC, blastocysts were assessed morphologically for grade, as described previously in Chapter 2 (Section 2.2). Also, the zona pellucida were removed from cleaved embryos and blastocysts using Pronase 0.2% for around 1 min, washed and kept individually in 2 µl PBS-PVP 0.1% at -20ºC for embryo sexing by real time PCR.

5.2.2 Effect of MG132 and CSF2 (8 h vs 18 h IVF) on embryo sex

A total of 278 embryos, including 65 blastocysts harvested at D8 of *in vitro* culture, were utilized in the analysis of primary sex ratio. Polymerase chain reaction (PCR) was performed to investigate the effect of MG132 and CSF2 on sex ratio of embryos and blastocysts at D8 of IVC. The end point of PCR was gel electrophoresis. Sex determination region Y (*SRY*) was selected from GenBank (NCBI) as a marker for the Y chromosome and forward and reverse primers were designed utilizing Primer Express software version 3.0.1. Bovine specific primer (BSP) was selected according to Rattanasuk et al. (2011) to identify the sequence (Table 1). Forward and reverse primers were supplied by Eurofins Genomics (GmbH, Anzinger Str. 7A, 85560, Ebersberg, Germany).

Table 5.1. Genes and selected primer sequences utilized for sex determination of bovine embryos and blastocysts.

Genes	Accession no.	Sequences (5'-3')
SRY	EU581861.1	F: 5'-TGAAACAA-GACCAAAACCGGG-3' R: 5'-TCCATGGACTTGCTCTACTGT-3'
BSP	Rattanasuk et al.,2011	F: 5'-TTTACCTTAGAACAAACCGAGGC-3' R:5'-TACGGAAAGGAAAGATGACCTGACC-3'

As a positive control, genomic DNA was extracted utilizing DNeasy kit (Qiagen, cat. 69504) according to the manufacturer's instruction (spin column protocol) (**Figure 5.1**). Male liver and granulosa cells were utilized for the detection of the related DNA sequence. Briefly, 20 mg liver tissue was homogenised in 1.5 microcentrifuge tube containing 180 µl ATL buffer and 20 µl proteinase K mix and vortex for 15s followed by overnight incubation at 56°C for the tissue. For granulosa cells, less than $5x10^6$ cells were centrifuged for 5 min at 300 x g to remove liquid and then the pellet was resuspended with 200 µl PBS and 20 µl proteinase K. After that, 4 µl RNase A (100 mg ml^{-1}) was added for both tissue and cells followed by incubation for 2 min at room temperature and vortexed for 15s. Then, 200 µl AL buffer was added and mixed by vortex followed by 10 min incubation at 56°C for the cultured cells. 200 µl absolute ethanol (96-100%) was added and mixed by vortex. This solution was then transferred with precipitate to DNeasy spin column followed by centrifugation at ≥ 6000 x g for 1 min. The spin column was transferred to a new 2 ml tube and 500 µl AW1 buffer was added followed by the mix and centrifugation at ≥ 6000 x g for 1 min. Then the Spin column was transferred to new a 2 ml tube and 500 µl AW2 buffer was added followed by centrifugation at 20,000 x g for 3 min. The column was then transferred to a new 1.5 ml microcentrifuge tube followed by spinning at maximum speed for 1 min. Then the column transferred to another new 1.5 ml microcentrifuge tube and left to dry for 2 min with open lid. 200 µl AE buffer were added to the column at room temperature for 1 min

followed by centrifugation at ≥ 6000 x g for 1 min. For determination of DNA concentration, 1.5 µl of eluted DNA were uploaded in Nanodrop and 1 ng gDNA was utilized in each PCR reaction.

Total PCR reaction volume was 25 µl containing, 12.5 µl Immomix Red (Bioline), 0.125 µl (10 pmol μl^{-1}) of forward and reverse primer of BSP, 1.25 µl (10 pmol μl^{-1}) of forward and reverse primer of SRY, 2 µl gDNA and 7.75 µl of PCR water.

Initial step at 95 ºC for 10 min was applied to all reaction tubes to lyse embryo and to activate enzyme. PCR was run for 38 cycles at three temperatures; denaturation at 94ºC for 30s, annealing at 55ºC for 30s and extension at 7 ºC for 1 min; then one cycle at 72ºC for 7 min and hold at 10ºC. PCR products were kept at -20 ºC or used directly in gel electrophoresis.

Gel electrophoresis was performed as previously described in Chapter 2 (Section 2.2.5). Briefly, 10 µl PCR products was mixed with 2 µl of 6x loading dye before loaded on 1.6% agarose containing 0.5 µg/ml ethidium bromide along with 100 bp DNA ladder (Promega). Negative controls (using water instead of DNA) were included in each PCR run. The resultant bands were visualized under UV transilluminator and images were captured by UVP GelDoc-IT imaging system.

5.2.3 Statistical Analysis

Statistical analyses were performed using the GenStat statistical package (17th Edition, VSN International, 2011). All proportion data were analysed using generalized linear regression models that assumed binomial errors and used logit-link functions. Analyses of cell number (inner cell mass and trophectoderm) used generalized linear regression models but assumed Poison errors and used log-link functions. Gene expression was analysed by analysis of variance (ANOVA).

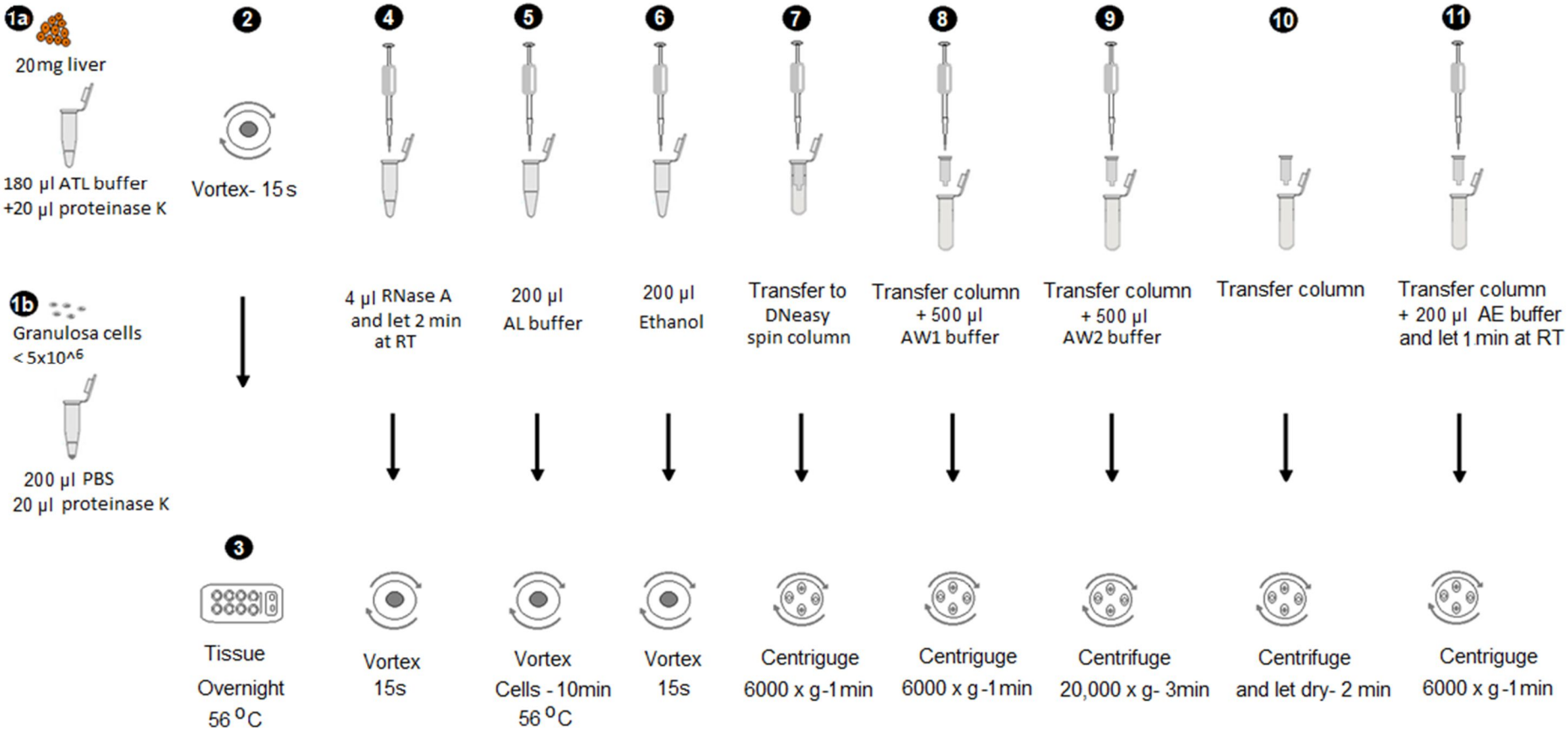

Figure 5.1. Illustration diagram for DNA extraction utilizing DNeasy kit and spin column protocol.

Residual plots confirmed that data were normally distributed and homogeneous. Data are presented as predicted means and standard error of these means (s.e.m). In each case, differences between individual means were established using Least Significant Differences (LSD) derived from residual errors of the statistical models. Differences were considered significant at P<0.05.

5.3 Results

5.3.1 Effect of MG132 and CSF2 (8 h vs 18 h IVF)

Incubating matured oocytes with sperm for 8 h reduced (P = 0.017) the proportion of cleaved zygotes at D2 of culture, but this effect was reversed if oocytes had been matured in the presence of MG132 (**Figure 5.2A**). On the other hand, co-incubation of gametes for 8 h increased (P<0.001) the proportion of ≥4 cell embryos on D2; an effect that was reduced when COCs had been matured with MG132 (**Figure 5.2B**).

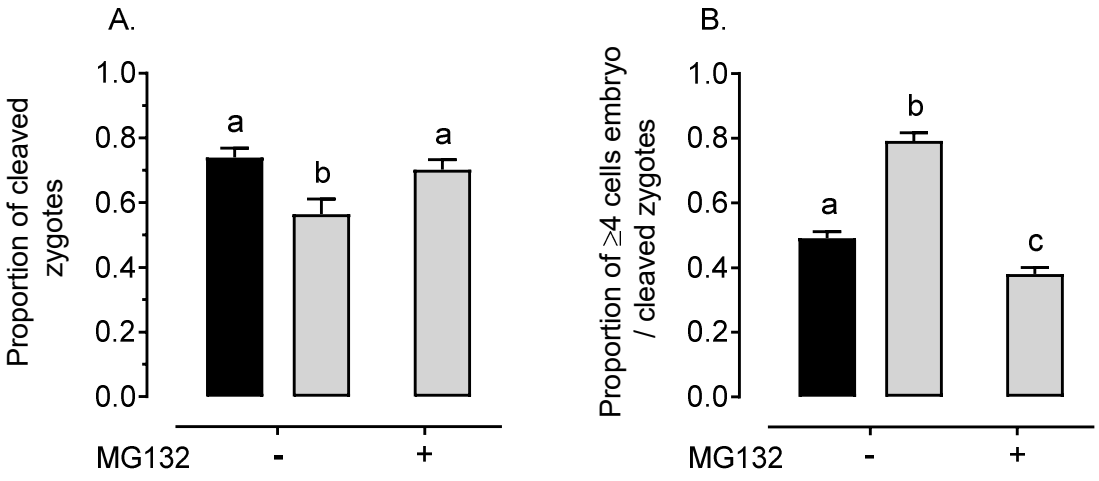

Figure 5.2. Proportion of cleaved zygotes (A), and ≥4 cell embryos from cleaved zygotes (B) on D2 of culture following 18 h (black bar) vs 8 h (grey bar) IVF for oocytes matured with 0 or 10 μM MG132. Means with different superscripts are different at P<0.05. Experiment was replicated four times.

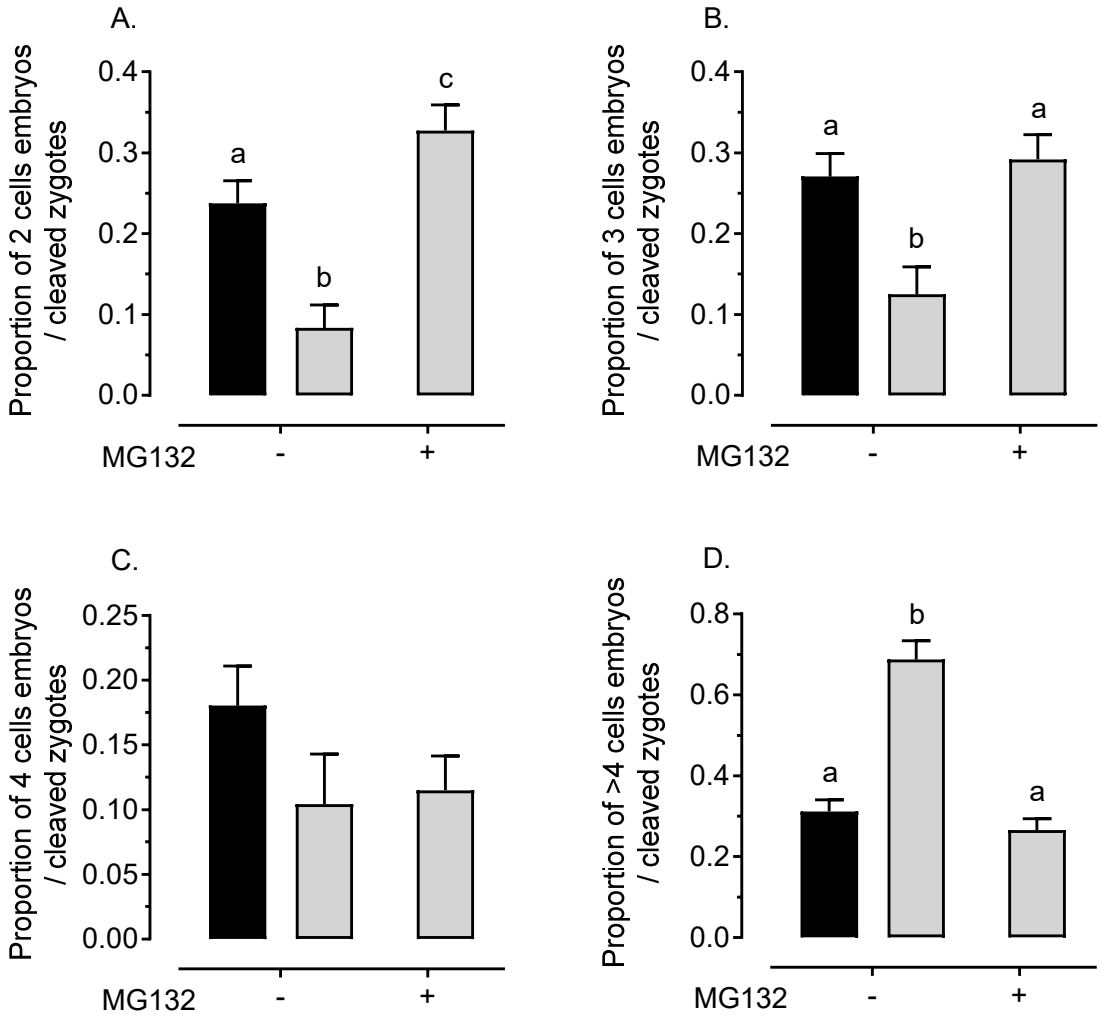

Figure 5.3. Proportion of embryos in different developmental stages at D2 of culture following 18 h (black bar) vs 8 h (grey bar) IVF for oocytes matured with 0 or 10 μM MG132. Means with different superscripts are different at P<0.05. Experiment was replicated four times.

In terms of subsequent embryonic development (i.e. blastocysts from cleaved zygotes), relative to the standard control (i.e. 18 h IVF with no added growth factors) all other treatment combinations had a beneficial effect (P = 0.039) (**Figure 5.4**). However, there was no apparent additive effect of combining MG132 with CSF2 in this experiment.

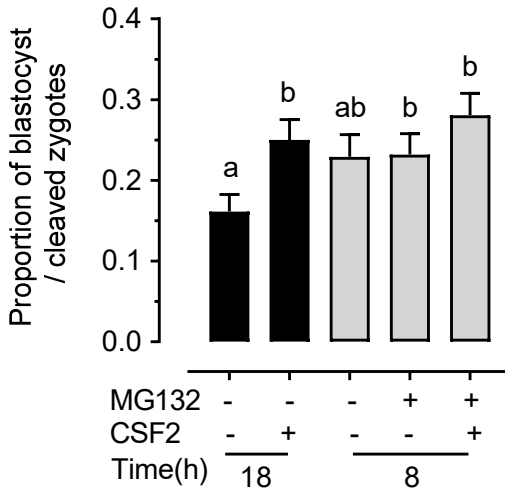

Figure 5.4. Blastocysts from cleaved zygotes following 18 h (black bar) vs 8 h (grey bar) IVF for oocytes matured with 0 or 10 μM MG132 and embryos treated with CSF2 from D5 of culture. Means with different superscripts are different at P<0.05. Experiment replicated four times.

5.3.2 Effect of MG132 and CSF2 (8 h vs 18 h IVF) on embryo sex

In terms of utilizing the PCR and gel electrophoresis for the determination of embryo gender (**Figure 5.5**), data showed that embryo sex ratio did not differ between treatment groups for the total embryos that harvested irrespective to the stage of development at D8 of IVC in comparison to the Control, 18 h group (**Figure 5.6A**). The sex ratio of blastocysts tend to shift sharply towards male in 8 h IVF control group. However, the combination of MG132 with CSF2 skew the sex ratio in blastocysts (P=0.05) towards female in comparison with the Control, 8h IVF group (**Figure 5.6B**).

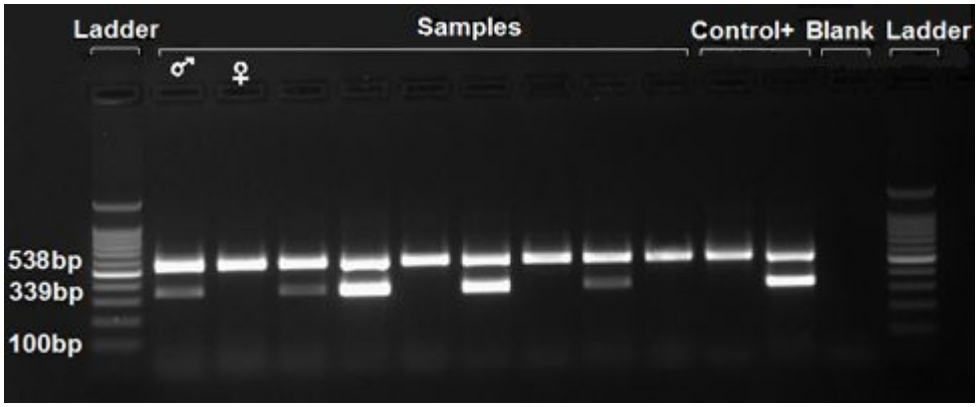

Figure 5.5. Gel electrophoresis for the detection of embryo sex harvested at D8 of *in vitro* culture and following 18 h vs 8 h IVF for oocytes matured with 0 or 10 µM MG132. Single band indicating female and double band indicating male.

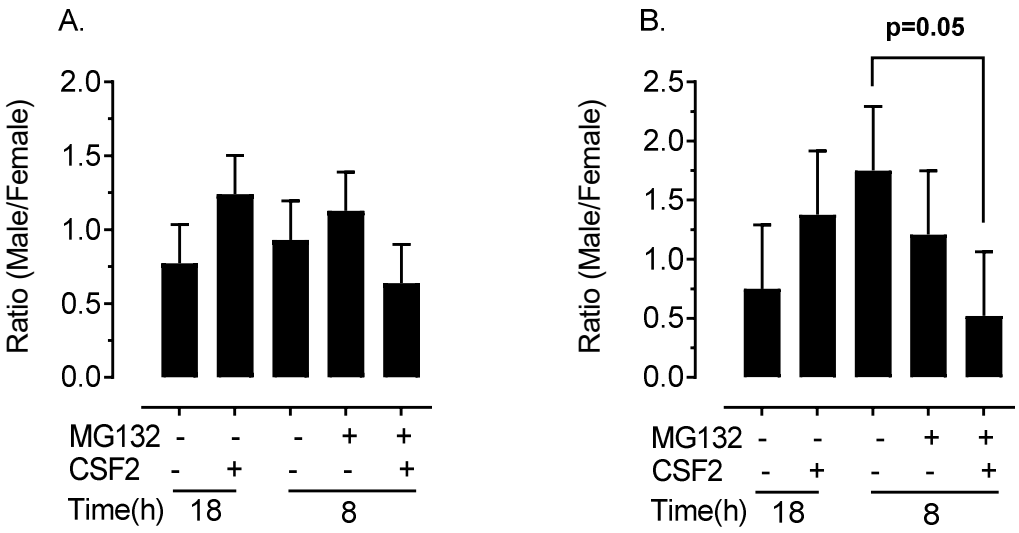

Figure 5.6. Sex ratio for total harvested embryos irrespective of the stage of development at D8 of IVC (A), and for total blastocysts (B) harvested at D8 of IVC following 18 h vs 8 h IVF for oocytes matured with 0 or 10 µM MG132, and/or embryos treated with CSF2 from D5 of culture. Experiment replicated four times.

5.4 Discussion

The current study was undertaken to investigate the effects of a short period of co-incubation of gametes, together with the effects of MG132 during late maturation and CSF2 from D5 of *in vitro* culture, on blastocyst yield and embryo sex. This study revealed that a short period of co-incubation of gametes reduces fertilization rates but that MG132 can overcome this deficit to return fertilization rates to those observed for conventional 18 h IVF. Also, blastocyst yields increased with MG132 followed by 8 h IVF. Also, a greater proportion of blastocysts were observed following treatment with CSF2. Embryo sex was not altered by treatment, averaging around 50:50 or 1:1 across treatment groups. However, in comparison to Control, 8 h group, MG132 with CSF2 following 8 h insemination tended to produce more female blastocyst.

5.4.1. Effect of MG132 and CSF2 (8 h vs 18 h IVF)

Consistent with the findings of Chapter 3 (Section 3.4.3), the results of this chapter showed that MG132 increases fertilization rate and early cleavage development following a short period of co-incubation of gametes. It has previously been demonstrated that MG132 has a beneficial effect on post-fertilization development (You et al., 2012; 2012a; Ono et al., 2011 and Xiao et al., 2014). In agreement with previous studies, CSF alone improved the development of embryos to the blastocyst stage (Loureiro et al., 2009; Neira et al., 2010; Dobbs et al., 2013 and Sequeira et al., 2017). The results of this study revealed, for the first time to our knowledge, that MG132 followed by 8 h IVF separately or in combination with CSF2 from D5 of culture produces more blastocysts than Control 18 h IVF. The mechanism by which MG132 improves the developmental efficiency of bovine embryos is still not established, but it was reported that MG132 upregulates a variety of proteins in bovine oocytes including glycolytic (GAPDH) and anti-apoptotic (P4HB) which have an anti-aging effect on oocytes leading to improvements their developmental competence (You et al., 2012a).

In relation to Control, 8 h group, CSF2 with MG132 had no effect on embryonic development to the blastocyst stage. It has been shown that CSF2 exhibits its stimulatory effect only when the level of embryonic development is low during *in vitro* culture (Dobbs et al., 2013). This means that in 8 h IVF regime, treatment with MG132 produced more developmentally competent embryos which was not different compared to the CSF2 treated groups from D5 of *in vitro* culture. As previously demonstrated in Chapter 3 (Section 3.4.3.), duration of IVF determined the effect with MG132 added late during maturation on post fertilization development of the bovine embryo. However, further studies with regard to the effect of MG132 during late maturation on bovine oocytes and on fertilization events, and particularly cyclin B degradation and pronuclear formation during the first hours post insemination, are required.

5.4.2. Effect of MG132 and CSF2 (8 h vs 18 h IVF) on embryo gender

In concordance with King et al., (1991) sex ratio was around 1:1 for the total population of embryos at D8 of *in vitro* culture for all Control 18 h and 8 h IVF groups. In the current study, a short incubation period of gametes for 8 h appeared to skew the sex ratio of bovine blastocysts toward male, but the effect wasn't statistically significant. On the other hand, in spite of the skewing of the sex ratio of blastocysts to male in the short 8 h IVF regime, data show no difference in comparison with 18 h IVF regime. It had been shown that short period of IVF resulted in low fertilization rates but a higher capacity of oocytes to develop to the blastocyst stage following fertilisation with a high male ratio in the resultant blastocysts (Kochhar et al., 2003). Moreover, during the culture of bovine embryos *in vitro*, male embryos developed more rapidly than female embryos to the blastocysts stage (Xu et al., 1992 and Green et al., 2016). It might be possible that low number of blastocysts utilized in this study affected the sex ratio of the resultant blastocysts in the short IVF regime. Further studies utilizing larger populations of blastocysts is recommended.

There is limited data with regard to the effects of CSF2 on sex ratio during *in vitro* culture. However, using sex sorted semen, treatment of bovine embryos from D5 of culture with CSF2 increased the development of female embryos to the blastocyst stage (Siqueira and Hansen, 2016). However, our results showed that, in 18 h IVF regime, treatment of embryos with CSF2 from D5 of IVC had no effect on sex ratio of blastocysts. However, in 8 h IVF regime, MG132 during late maturation and CSF2 from D5 of *in vitro* culture tended to produce more female blastocysts. As previously discussed, MG132 during late maturation followed by 8 h IVF increased fertilization rate but reduced >4 cell embryos by D2 of *in vitro* culture. Also, CSF2 increases blastocyst yields only when the level of development is low (Dobbs et al., 2013). It seems that MG132 reduces the level of development of male embryos in culture and CSF2 boosts the development of female embryos to the blastocyst stage in response to this reduction. This means that more female blastocysts could be produced from the combination of MG132 during late maturation followed by 8 h IVF and CSF2 from D5 of in vitro culture. However, this remains to be confirmed.

5.5 Conclusions

This is the first detailed study designed to investigate the effect of MG132 followed by 8 h IVF with CSF2 from D5 of culture on post-fertilization development of bovine embryos. Also, it is the first to investigate the effect of this treatment on sex ratio of embryos and blastocysts at D8 of culture. The current study revealed that a short period of co-incubation of gametes produces fewer fertilized zygotes, but those that are produced are relatively well-developed. In contrast, MG132 increases fertilization rate and produces more competent zygotes that proceed to the blastocyst stage. Also, CSF2 from D5 of culture improves the development of zygotes to the blastocyst stage under the 18 h IVF regime. On the other hand, MG132 during late maturation, followed by 8 h IVF and CSF2 from D5 of culture increases blastocyst yield at D8 of culture and tended to alter sex ratio of the resultant blastocysts in favour of females. This latter finding has a significant

implication on future IVP research and bovine embryo production. However, the effect of MG132 during the first hours of fertilization and its role in increasing fertilization post a short period of gamete co-incubation requires further investigation. Also, due to the scarcity of the research in terms of utilizing CSF2 in relation to embryo sex, more studies in this area are required. Moreover, further studies with regards to embryo survival post transfer utilizing MG132 during late maturation followed by a short period gamete co-incubation with CSF2 from D5 of culture is recommended.

6.1 General Discussion

6.1.1 Effect of SNP and MG132 during *in vitro* maturation

This series of experiments was designed to establish the effect of a combination of SNP and MG132 on nuclear maturation and post fertilization development of bovine embryos (Section 2.3.1 to 2.3.5 Chapter 2). The purpose of this combination of reagents was to maintain sufficient mitochondrial DNA copy number and produce competent oocytes that resulted in successful post fertilization development. Few studies have tested the effects of SNP administered during maturation on subsequent embryonic development (Viana et al., 2007). Unexpectedly, SNP separately and in combination with MG132 reduced polar body extrusion in matured oocytes (64% and 50%) compared to the non-treated group (86%) but had no effect on ATP content, mtDNA copy number, and post fertilization development of zygotes (**Figure 2.10**, to **Figure 2.17**). The reduction of polar body extrusion might be attributed to the effect of SNP on sustaining certain levels of cGMP that delays the transition of some oocytes beyond meiosis (**Figure 6.1**).

On the other hand, surprisingly, MG132 treatment late in maturation reduced first polar body extrusion to 61%. Other studies reported that cyclin B degradation by proteasomes is an essential mechanism by which the oocyte progresses meiosis to anaphase (Holloway et al., 1993; Josefsburg et al., 2000; Peters J., 2002; Chang et al., 2003; Herbert et al., 2003; Parry et al., 2003 and Hyslop et al., 2004). Consequently, MG132 administered during late maturation delays cell cycle progression and first polar body extrusion which could be mediated through abundance of cyclin B (**Figure 6.1**).

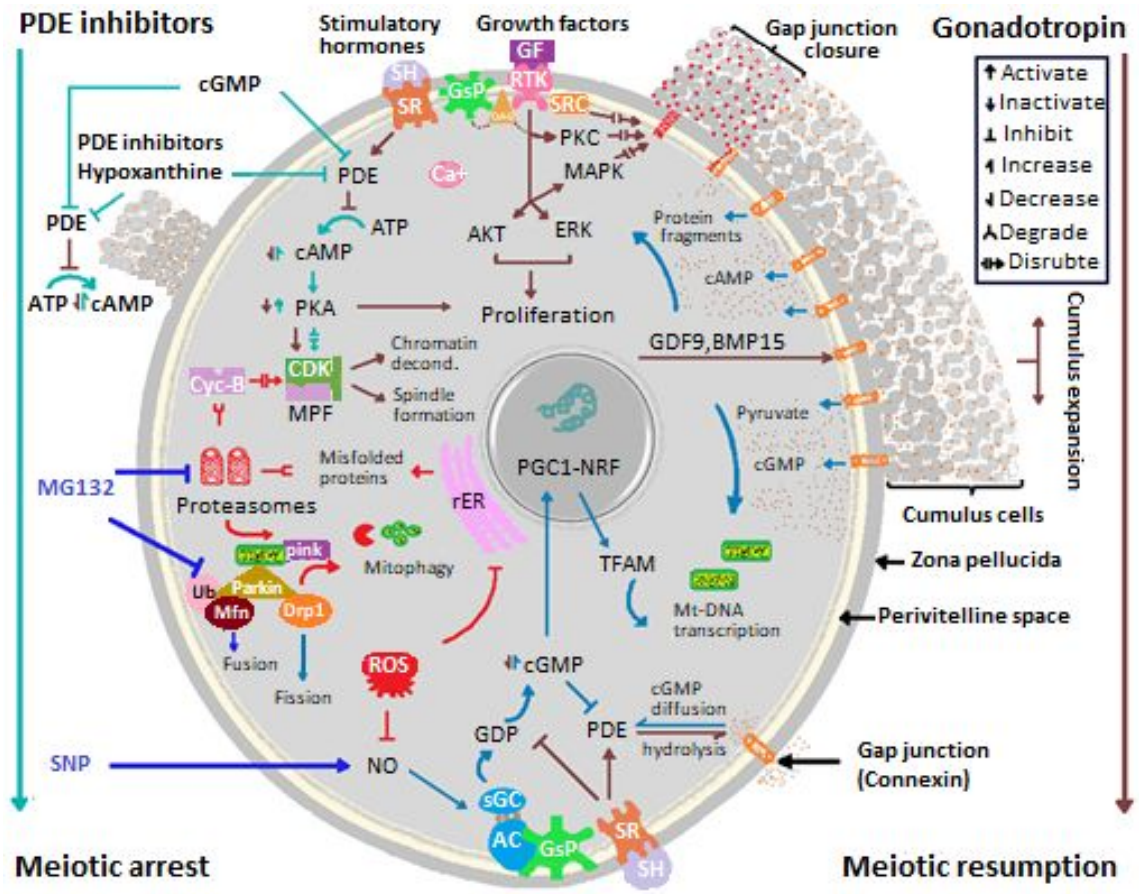

Figure 6.1. Proposed effect of SNP on the elevation of cGMP and the mechanism by which stimulatory hormones inhibit the formation of cGMP and the pivotal effect of MG132 during meiosis on proteasomal degradation particularly on cyclin-B that affecting the level of maturation promoting factor (MPF) and on mitofusin (Mfn) that interfering mitochondrial fusion and mitophagy.

MG132 added late during maturation separately or in combination with SNP reduced ATP content in oocytes relative to the non-treated group (0.96± 0.090 and 1.04± 0.097 vs 1.59± 0.135). Whereas cellular demand and consumption affects ATP levels (Dalton et al., 2014). Proteasomes play an essential role in balancing the process of protein synthesis and degradation (Martinez-Vicente et al., 2005 and Rothman, 2010), and the dependency of the 19s regulatory particle of proteasome and ubiquitination reaction on ATP (Yi et al., 2009 and Saez and Vilchez, 2014). Consequently, this led us to the conclusion that the decline of ATP in oocytes observed in this study following treatment with MG132 during late maturation is related to the reduction of ATP demand for protein degradation and synthesis (**Figure 6.2**).

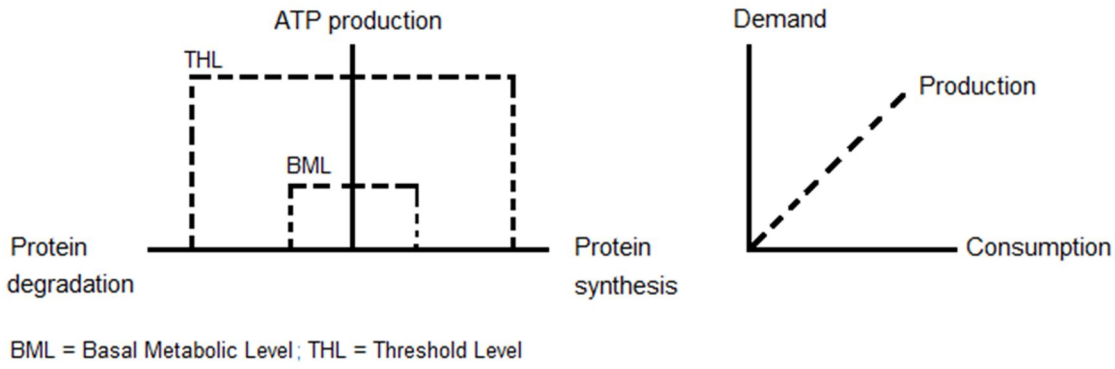

Figure 6.2. Proposed relationship between ATP production and protein degradation and synthesis. Increase ATP demand and consumption lead to increase ATP production. ATP production depend mainly on the balance between synthesis and degradation of proteins.

On the other hand, mtDNA copy number variation post treatment with SNP and MG132, separately or in combination, could be due in part to differences in animal age (Iwata et al., 2011 and Takeo et al., 2013). Natriuretic peptide receptor 2 (NPR2) is activated by C- natriuretic peptide (CNP) and is involved in the production of cGMP. Also, the LH surge decreases the level of CNP that limits the conversion of GDP to cGMP (Kawamura et al., 2011). Collectively, these effects could explain why treatment with SNP during maturation is not sufficient to increase mtDNA copy number in the presence of stimulatory hormones in maturation medium. Moreover, MG132 during late maturation had no effect on mtDNA copy of matured bovine oocytes. In other studies, oocytes harvested from young cows and treated with 10 µM MG132 for 21 h during *in vitro* maturation increased mtDNA copy number (Kansaku et al., 2017). This might raise a question for further investigation of the possible role of age in the variation of mtDNA copy number following short-term treatment with MG132 during late maturation.

MG132 separately or in combination with SNP, followed by incubation of gametes for 18 h during fertilization, reduces the development of zygotes reaching the blastocysts stage. MG132 alone or in combination with SNP reduced cleavage in comparison to the non-treated group (78% and 68% vs 84%), as well as the proportion blastocysts from cleaved

zygotes (11% and 4% vs 19%). Other studies have reported that utilizing MG132 followed by 8 h incubation of gametes during fertilization has a beneficial effect on embryonic development (You et al., 2012a). Active mitochondria in cumulus cells have a crucial role on oocyte development and affect fertilization outcomes (Dumesic et al., 2016 and Gorshinova et al., 2017), in part by providing extracellular source of ATP during fertilization (Yi et al., 2009). Therefore, reduction of ATP in the oocyte in response to treatment with MG132 could be attributed in part to its residual effect in cumulus cells which results in defective post-fertilization development.

6.1.2 Effect of MG132 dose and timing of gamete incubation

The purpose of this study was to investigate the effect of different concentrations of MG132 with a truncated IVF regime on the development of bovine embryos (Section 3.3.1 and 3.3.3 Chapter 3). Results indicate that 10 µM MG132 followed by 18 h IVF reduces the proportion of well-developed embryos at D2 compared to the non-treated group (9% vs 23%) (**Figure 3.3**). Similar to previous findings, short incubation of gametes resulted in a reduction of the proportion that cleaved (Ward et al., 2002 and Barraud-Lange et al., 2008). However, cleavage was increased post treatment with MG132 followed by 8 h IVF compared to non-treated group in 18 h IVF system (72% vs 60%) (**Figure 3.8**). The percentage of well-developed embryos at D2 was also increased compared to treated counterpart (55% vs 26%) (**Figure 3.10B**). Long-term incubation of sperm with MG132 treated COCs during late maturation reduced ATP required for post fertilization events and could be behind the retardation of embryonic development (Sumantri et al., 1997; Takahashi et al., 2000; Liu et al., 2001; Gordo et al., 2002; Ward et al., 2002; Kochhar et al., 2003; Igarashi et al., 2005; Iwata et al., 2008; Enkhmaa et al., 2009; Lopes et al., 2010 and Darbandi et al., 2016). Moreover, cyclin B degradation is an ATP dependent process and necessary to overcome MII arrest (Marangos and Caroll, 2004 and Darbandi et al., 2016). Thus, and in accord with previous studies, a

truncated IVF regime is beneficial to embryonic development when applying MG132 during late maturation (You et al., 2012a and Xiao et al., 2014) (**Figure 6.3**).

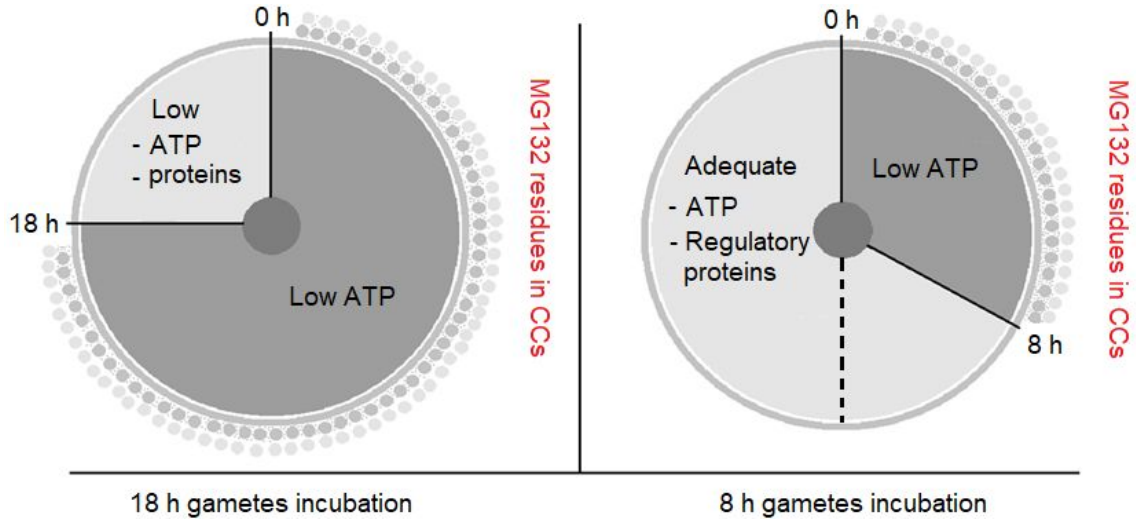

Figure 6.3. Schematic illustration of the proposed residual effect of MG132 in cumulus cells and the effect of incubation period of gametes on ATP and essential regulatory proteins post fertilization. Note that in 18 h IVF regime ATP production is reduced as a response to MG132 residues in cumulus cells that persists longer than 8 h IVF.

Another interfering mechanism by which MG132 might exert its negative residual effect during conventional 18 h IVF is by actions on gap-junction connexin levels during maturation. In a previous study, connexin degradation was reduced by administration of MG132 (Huang et al., 2010a and Nagyova, 2018). ATP may be reduced in cumulus cells after treatment with MG123, which could result in the reduction of ATP flux through gap junctions from cumulus cells and hence reduced ATP levels in oocytes (Wang et al., 2009). This in turn indicates that treatment of COCs with MG132 during maturation may delay the closure of gap junctions but has no beneficial effect on ATP flux to oocytes from cumulus cells because of reduced levels. However, ATP reduction could be rescued by early denudation of oocytes from cumulus cells in order to eliminate the long-term residual effect of MG132 in those cells.

6.1.3 Effect of TGFB1 and CSF1

This study sought to investigate the effect of a combination of TGFB1 and CSF2 from D5 of *in vitro* culture (Section 4.3.1 and 4.3.2 Chapter 4). The purpose behind selecting these two factors lay in their important effects on embryonic development with no previous reports for the use of this combination during culture (Neira et al., 2010; Dobbs et al., 2013 and Sequeira et al., 2017). In the current study TGFB1 reduced blastocyst yields that would otherwise be increased with CSF2 compared to the non-treated group (10% vs 20%) (**Figure 4.4**). However, TGFB1 separately from D5 or D2-5 had no effect on the development of bovine embryos at the current concentration. It might be possible that high concentrations of TGFB1 might be required to exert its beneficial effects during preimplantation (Keefer, 1992; Neira et al., 2010 and Moreno et al., 2015). Also, compared to the non-treated group, CSF2 individually increased blastocyst yields (25% vs 13%) (**Figure 4.6**) and the number of cells within the inner cell mass in early (26 vs 20) and late blastocyst (18 vs 14) (**Figure 4.10**). In another supporting study, CSF2 increased the number of cells within the inner cell mass in bovine blastocysts at D8 of IVC (Loureiro et al., 2009).

The variability in the effect of CSF2 on the proportion of blastocysts between experiments might be attributed to differences in the total developmental potential of embryos between experiments. It has been demonstrated that CSF2 increases blastocyst yields only when the level of embryonic development is low in culture (Dobbs et al. 2013). On the other hand, investigating the effect of TGFB1 on *SMAD* canonical signalling pathways indicates that 10 or 50 ng ml^{-1} TGFB1 had no effect on *SMAD2* and *SMAD3* expression on embryos at D7 of IVC (**Figure 4.12**). Whereas diminishing SMAD signals after the 8-cell stage during bovine embryo development had been demonstrated (Zhang et al., 2015). Therefore, TGFB1 acts by a non-canonical pathway to eliminate the stimulatory effect of CSF2 on the development of bovine embryos cultured *in vitro.* However, the precise mode of action of TGFB1 interference on CSF2 actions from D5

on bovine embryo development remains to be fully determined. However, TGFB1 is known to inhibit PIK3 which is stimulated by CSF2 and leads to the suppression proliferation of human leukemic cells (Montenegro et al. 2009). This is worthy of further study in bovine embryos.

6.1.4 Effect of MG132 followed by 8 h IVF and CSF2 in culture

The purpose of this study was to investigate the effect of the best combination treatments from previous chapters on the development and primary sex ratio of bovine blastocysts (Section 5.3.1 and 5.3.2 Chapter 5). In this study, short incubation of gametes for 8 h reduced fertilization and administration of MG132 in late maturation neutralized this proportion compared to the non-treated group in the 18 h IVF regime (**Figure 5.2A**). The percentage of well-developed embryos at D2 was reduced post treatment with MG132 compared to the non-treated group in 18 h IVF system (38% vs 49%) (**Figure 5.2B**). Also, the blastocyst proportion was increased post administration of MG132 in late maturation and CSF2 from D5 compared to non-treated group in 18 IVF regime (**Figure 5.4**). In previous studies, MG132 during late maturation had a beneficial effect on the development of mammalian embryos through abundance of regulatory proteins involved in glycolysis and apoptosis (You et al., 2012, 2012a; Ono et al., 2011 and Xiao et al., 2014). Also, CSF2 improved the development of bovine embryos during late preimplantation development (Dobbs et al., 2013 and Sequeira et al., 2017). On the other hand, sex ratio of embryos harvested at D8 of *in vitro* culture irrespective of stage was not different, and was around 50:50 (**Figure 5.6A**). However, treatment with MG132 followed by 8 h IVF and CSF2 from D5 of IVC shifted the sex ration towards female compared to the non-treated counterpart (0.52 vs 1.75) (**Figure 5.6B**). It has been shown that truncated IVF results in low fertilization rates but with more male blastocysts (Kochhar et al., 2003). Also, in one study, utilizing sex sorted semen, CSF2 increases the development of female bovine embryos cultured *in vitro* (Siqueira and Hansen, 2016). It was also reported that CSF2 has an effect on genes related to pluripotency and

differentiation resulting in enhancement of embryonic development *in vitro* (Hansen et al., 2014a).

MG132 during late maturation followed by 8 h IVF increases fertilization rate but reduces well developed embryos at D2 of *in vitro* culture. On the other hand, CSF2 enhances the development of embryos to blastocysts only when the level of development is low in culture (Dobbs et al., 2013). While in this study MG132 reduces the proportion of well-developed embryos in culture, CSF2 increases the development of female embryos to the blastocyst stage in response to this reduction. This approach might have a significant implication in IVP research in cattle raising the possibility of increasing the proportion of female embryos in future.

6.2. General conclusions

Results of the current series of studies reveal that the proteasome inhibitor (MG132) and nitric oxide donor (SNP) can delay the ability of oocytes to resume meiosis at the end of *in vitro* maturation. Also, MG132, but not SNP, reduces ATP content and post fertilization development of bovine embryos cultured *in vitro*. Moreover, MG132 had a beneficial effect on the number of zygotes reaching the blastocyst stage but only if followed with a short incubation of gametes during IVF. The residual effect of 10 μM MG132 in the remnant cumulus cells with 18 h IVF retards the development of zygotes by D2 and reduces blastocyst yields by D8 of IVC. Also, a short period of incubation of gametes for fertilization resulted in fewer fertilized but a higher developmental potential embryos from D2 of culture. However, administration of MG132 during late maturation increases the number of cleaved zygotes at D2 in short IVF regime.

Regarding later stages of embryo development, TGFB1 at the concentration used in the current study had no effect on the development of embryos beyond D5 of *in vitro* culture, or on cell number in the resultant blastocysts. However, CSF2 increases blastocyst yields at D8 and number of cells within the inner cell mass of resultant early and late

blastocysts. TGFB1 eliminates the stimulatory effect of CSF2 when combined from D5 of IVC. Moreover, this effect of TGFB1 is not a response to the effect of the canonical *SMAD* signaling pathway, rather it involves non-canonical signaling pathways that interfere with the proliferative effect of CSF2.

Adopting a system that utilizes MG132 during late IVM followed by short IVF with the administration of CSF2 from D5 resulted in high blastocyst yields at D8 of IVC. Moreover, this system resulted in the skew in the sex ratio of females in resultant blastocysts.

6.3. Future studies

MG132 improves the developmental competence of oocytes but only with early denudation of cumulus cells after short IVF. It is therefore necessary to further investigate the role of cumulus cells during fertilization. We focused in our studies on the gross effects of MG132 during late maturation of bovine oocytes. However, further investigation is required to identify the precise mechanism of action of MG132 on cumulus cells during the process of fertilization and on cyclin B levels, ATP content and mtDNA copy number in oocyte in relation to the age of animal. Another suggested approach is reducing the period of time of treatment with MG132 and starting when the majority of oocytes show nuclear maturation and polar body extrusion (post 19 h maturation instead of 16 h).

In the present study, TGFB1 did not change the expression of *SMAD* signaling pathway of bovine embryos at D7 of IVC. This raises important questions regarding roles of non-canonical signaling of TGFB1 on the stimulatory action of CSF2. Also, this cytostatic effect of TGFB1 might have important implications in cancer research. Therefore, investigating the non-canonical signaling pathways post treatment with TGFB1 during preimplantation is recommended. Whereas in the current study, a dose of 10 ng ml $^{-1}$

TGFB1 is utilized without affecting embryonic development. Consequently utilizing higher concentration than 10 ng ml $^{-1}$ of TGFB1 with optimizing the dosage is required.

On the other hand, there is limited information regarding the effect of CSF2 on sex ratio of blastocysts. Our study provides evidence that sex ratio of blastocysts is skewed towards females when MG132 is used during late maturation followed by a short period IVF and administration of CSF2 from D5 of IVC. However, future studies with the involvement of greater numbers of blastocysts is recommended due to the important implications of this for the cattle industry, and which might also play a crucial role in clinical application of human *in vitro* embryo production. Moreover, further studies with regards to embryo survivability after transfer utilizing MG132 during late maturation followed by short incubation of gametes for fertilization and CSF2 at D5 of *in vitro* culture is recommended in order to determine pregnancy outcomes.

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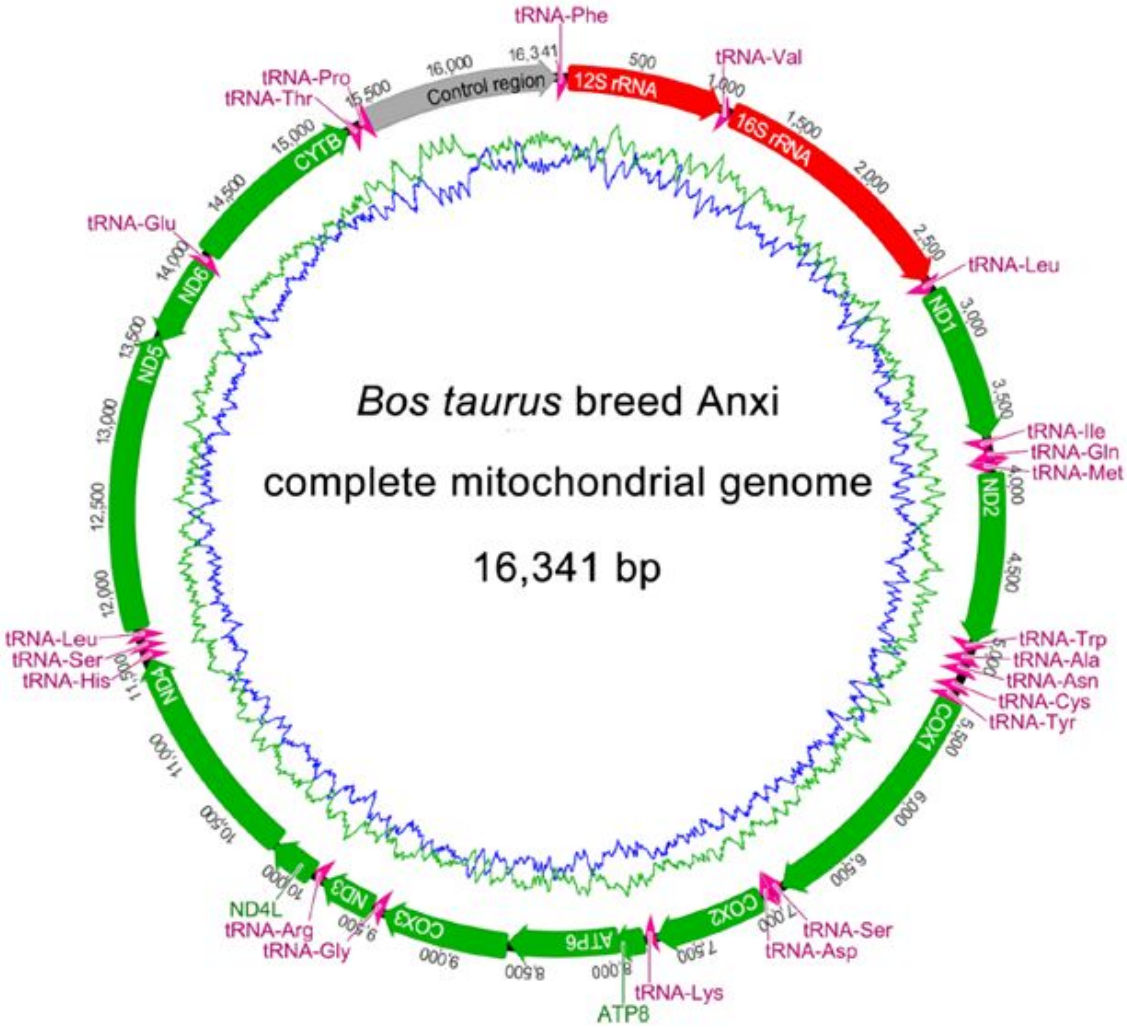
Appendices

Appendix – I – Complete mitochondrial genome



Appendix I - Complete mitochondrial genome *of Bos taurus* breed of Chinese Anxi cow. Source: Guo et al. (2017).

Appendix – II – Grading of COCs and blastocyst

A-

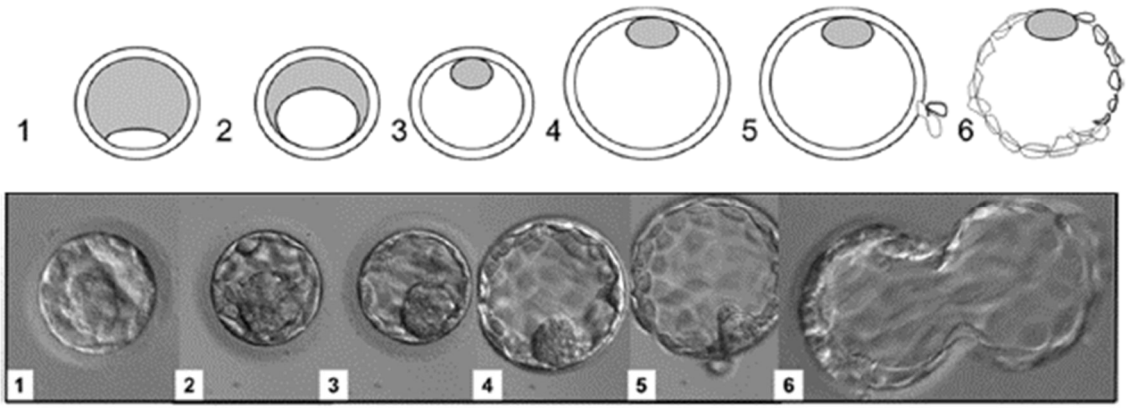
Morphological characteristics of Grade 1 and Grade 2 Cumulus oocyte complexes (COCs) utilized in the current study and according to Hazeleger et al. (1995).

Grade	Characteristics
(Grade 1)	-Compact and complete cumulus layer surrounding an oocyte with a homogenous ooplasm consisting of fine granulation and that is medium brown in color -oocyte > 120 µm in diameter.
(Grade 2)	-Compact and complete cumulus layer -The ooplasm is slightly more coarse than that of Group 1, with a dark zone around the periphery, medium brown in color -oocyte > 120 µm in diameter.

B-

Morphological classification and characteristics of bovine blastocysts. Based on six grade scoring system by Linder and Wright (1983). Also, on Gardner and Schoolcraft (1999) that depends on blastocoel size, zona pellucida thickness and the size of embryo (Reviewed by Kovacic and Vlaisavljevic, 2012).

Group	Characteristics
Early blastocyst	-Blastocoel less, equal or > than 50% of embryo, no zona pellucida thinning. No expansion of blastocyst.
Late blastocyst	-Blastocoel > 50% of embryo, zona pellucida thinning and size of embryo is greater than in early blastocyst.



Appendix - III – Medium and stocks

A- Ovarian transportation Medium- Phosphate buffered saline (PBS)

Component	g/ l	M
NaCl	8	0.1369
KCl	0.2	0.0027
Na_2HPO_4	1.44	0.0101
KH_2PO_4	0.24	0.0018

Medium pH adjusted to 7.4 and autoclaved for 20 min at 15 lb/in2

B- Oocyte washing Medium (HEPES-TALP)

Component	mg/ 100 ml	mM
NaCl	666	113.96
KCl	23.8	3.19
$NaHCO_3$	16.8	2
Na_2HPO_4	4.77	0.4
$MgCl_2 \cdot 6H_2O$	10	0.49
$CaCl_2 \cdot 2H_2O$	29.4	2
HEPES	240	10.07
Phenol Red (0.5%)	200 µl	0.028
Na-Pyruvate	5.5	0.5
Na-Lactate	300 µl	16.05
BSA	300	3 mg/ml
Pen/Strep	500 µl	50units/0.05mg/ml

Medium pH adjusted to 7.4 then Osmolarity 270-290 mOsm then the media sterilized by filtration through a 0.22 µm Millipore filter and equilibrated in 5% CO2 and air under 38.5ºC for at least 2 h prior to use.

C-Fert-TALP medium (Fertilization medium)

Component	mg/ 100 ml	mM
NaCl	544	93.09
KCl	23.8	3.09
$NaHCO_3$	220	26.19
$Na_2HPO_4 \cdot 2H_2O$	4	0.22
$MgCl_2 \cdot 6H_2O$	10	0.492
$CaCl_2 \cdot 2H_2O$	78	5.31
Caffeine	27	1.39
Na-Pyruvate	100	9.1
Na-Lactate	186 µl	9.95
BSA	600	6 mg/ml
Pen/Strep	500 µl	50units/0.05mg/ml
Heparin	100 µl	Stock 10 mg/ml
Hypotaurine	50 µl	Stock 1 mg/ml
Epinephrine	50 µl	Stock 1 mg/ml

Medium pH adjusted to 7.8 then Osmolarity 270-290 mOsm then media sterilised by filtration through a 0.22µm Millipore filter and equilibrated in 5% CO2 and air under 38.5ºC for at least 2 h prior to use.

D. Heparin Stock (10 mg/ml)

10 mg Heparin from porcine intestinal mucosa as a sodium salt (H3149)

1ml ddH_2O

Stock solution sterilized by filtration through a 0.22 µm Millipore filter, aliquots, and stored at -20ºC. Heparin stock was diluted in Fert-TALP media so that the final conc. is 10 µg/ml.

E. Epinephrine stock (1 mg/ml)

10 mg Epinephrine (H1384)

10 ml ddH_2O

Stock solution sterilized by filtration through a 0.22 µm Millipore filter, aliquots, and stored at -20ºC. Heparin stock was diluted in Fert-TALP media so that the final conc. is 0.5 µg/ml

F. Hypotaurine stock (1 mg/ml)

10 mg Hypotaurine (H1384)

10 ml ddH_2O

Stock solution sterilized by filtration through a 0.22 µm Millipore filter, aliquots, and stored at -20ºC. Heparin stock was diluted in Fert-TALP media so that the final conc. is 0.5 µg/ml.

G- Modified Synthetic oviductal fluid medium (mSOF medium)

Component	mg/ 100 ml	mM
NaCl	629	107.63
KCl	53.4	7.16
$NaHCO_3$	210	25
KH_2PO_4	16.2	1.19
$MgSO_4$	18.2	1.51
$CaCl_2 \cdot 2H_2O$	26.2	1.78
L-Glutamine	2.9	0.20
Tri-sodium citrate	10	0.34
Na-Pyruvate	80	7.27
Na-Lactate	75.7 µl	4.05
BSA	300	3 mg/ml
Phenol red (0.5%)	200 µl	0.028
Myo-Inositol	49.9	2.77
BME (50x)	4.5 ml	---
MEM (100x)	500 µl	---

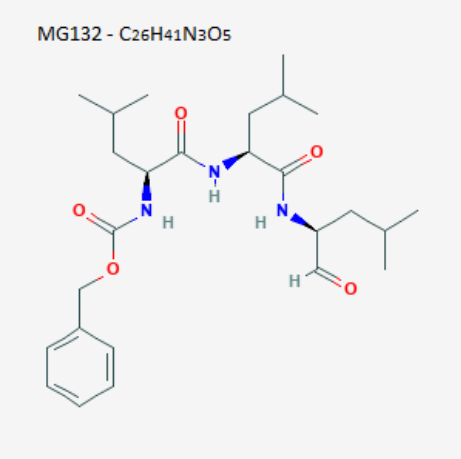
Medium pH adjusted to 7.4 then Osmolarity 270-290 mOsm. The medium filtered through a 0.22 µm Millipore filter and equilibrated in 5% O2, 5% CO2 and 90% N2 at 38.5ºC for at least 2 h prior to use.

H- HEPES synthetic oviductal fluid (hSOF)

Component	mg/ 100 ml	mM
NaCl	629	107.63
KCl	53.4	7.16
$NaHCO_3$	42	5
KH_2PO_4	16.2	1.19
$MgSO_4$	18.2	1.51
$CaCl_2 \cdot 2H_2O$	26.2	1.78
L-Glutamine	2.9	0.20
Tri-sodium citrate	10	0.34
Na-Pyruvate	80	7.27
Na-Lactate	75.7 µl	4.05
BSA	300	3 mg/ml
Phenol red (0.5%)	200 µl	0.028
HEPES	47.66	2
MEM (100x)	500 µl	---

Medium pH adjusted to 7.4 then Osmolarity 270-290 mOsm. The medium filtered through a 0.22 µm Millipore filter and equilibrated in 5% O2, 5% CO2 and 90% N2 at 38.5ºC for at least 2 h prior to use.

I. Proteasome inhibitor - MG132 stock (10mM)

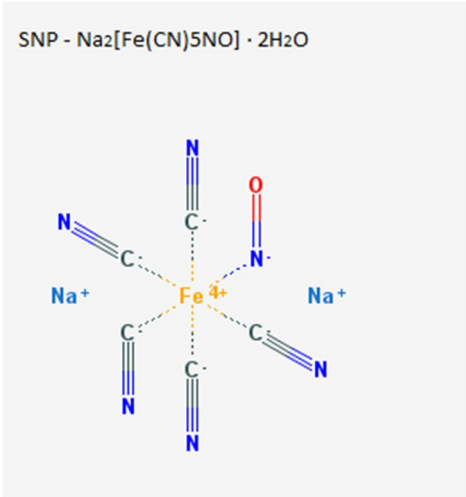


5 mg MG132 - C2211

2.103 ml DMSO - D2438

Mixing by vortexing then filtered with 0.22 µm Millipore filter and aliquated into 10 ul and store at -20ºC. Add in a volume of 2 µl / 1ml of maturation medium equivalent to 10 µM MG132.

J. Sodium nitroprusside - SNP stock (1mM)



5 mg SNP - 228710

16.78 ml ddH_2O

Mixing by vortexing then filtered with 0.22 µm Millipore filter. Prepared only fresh. Add in a volume of 10 µl / 1 ml of maturation medium.

K. GM-CSF stock (250 µg / ml)

25 µg GM-CSF - RP0871B-025

100 µl mSOF medium

Add 100 µl of sterile mSOF under hood. Mix by vortexing then aliquot into 10 µl and store at -20ºC. When use add 2 µl (500 ng) of stock to 1ml mSOF medium vortex well then transfer 100 µl of this to another 1ml mSOF medium to have the final concentration of 50 ng/ml.

L. TGFB1 stock (5 µg / ml)

5 µg TGFB1 – H8541

1 ml HCl 4mM containing 0.1% BSA

Firstly, centrifuge the vial prior to opining. After filtration of HCl 4mM-0.1%BSA add to vial and then mix by vortex. Aliquot into 25 µl and store at -20ºC. When use add 2 µl (10

ng) of stock to 1ml mSOF medium and vortex well to have the final concentration of 10 ng/ml.

M. Hoechst 33258 Stock (10 mg/ml)

20 mg Hoechst 33258 (B2883)

2 ml ddH_2O

Stock solution was aliquated into 10 µl volume and stored at -20ºC.

N. Hoechst 33342 Stock (5 mg/ml)

25 mg Hoechst 33352 (B2261)

5 ml ddH_2O

Stock solution was aliquated into 10 µl volume and stored at -20ºC. 10 µg/ml was prepared by adding 2 µl of Hoechst 33352 stock to 1ml TCM199 medium and utilized for 10 min prior fixation of zygote with 4% paraformaldehyde for 10 min and permeabilization with Triton-X100 (0.5% in PBS) for 1 h.