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Role of melatonin in bovine gametes competence and preimplantation embryo development *in vitro*

THESIS

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Dedicated to

My children

God has created you for a unique and wonderful purpose,

Only you can discover it, and the world is waiting to see it; hence,

Do never stop pursuing your dreams; they are the frame of that purpose.

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ABBREVIATIONS

AADC	**a**romatic L-**a**mino **a**cid **d**ecarboxylase
AANAT	**a**rylalkyl**a**mine *N*-**a**cetyl **t**ransferase
ACTB	**Act**in **b**eta
AFMK	2-hydroxylmelatonin, 6-hydroxylmelatonin, cyclic 3-hydroxymelatonin, N^1-**a**cetyl-N^2-**f**ormyl-5-**m**ethoxy**k**ynuramine
AJ	**a**dherens **j**unctions
ALH	**A**mplitude of **l**ateral **h**ead displacement
AMK	N^1-**a**cetyl-5-**m**ethoxy**k**ynuramine
AQP	**aq**ua**p**orins
AQP3	**aq**ua**p**orin **3**
ART	**a**ssisted **r**eproductive **t**echnologies
ASMT	*N*-**a**cetyl**s**erotonin *O*-**m**ethyl**t**ransferase
ATP	**a**denosine **t**ri**p**hosphate
ATP1A1	**ATP**ase Na+/K+ Transporting Subunit **A**lpha **1**
ATP1B1	**ATP**ase Na+/K+ Transporting Subunit **B**eta **1**
ATPase	Na+-K+-**a**denosine **t**riphos**p**hat**ase** pump
BAD	**B**CL2 **A**ssociated Agonist of Cell **D**eath
BAX	**B**-cell leukemia/lymphoma 2 **A**ssociated **X** Apoptosis Regulator
BCF	**b**eat **c**ross **f**requency
Bcl-2	**B**-**c**ell **l**eukemia/lymphoma **2**
BSA	**b**ovine **s**ynthetic **a**lbumin
C-3HOM	**c**yclic **3**-**h**ydr**o**xy**m**elatonin
Ca^{2+}	**ca**lcium
CASA	**c**omputer-**a**ssisted **s**perm **a**nalysis
Casp3	**casp**ase **3**
CAT	**cat**alase
CDH1	epithelial **c**a**dh**erin 1
cDNA	**c**omplementary **d**eoxyribo**n**ucleic **a**cid
CDX2	**c**au**d**al type homeobo**x** transcription factor **2**
CO_2	carbon dioxide
COCs	**c**umulus-**o**ocyte **c**omplexe**s**
CR1	**C**harles **R**osenkrans medium **1**
DAP	**d**istance **a**verage **p**ath
DCL	**d**istance **c**urvi**l**inear
DMSO	**d**i**m**ethyl **S**ulf**o**xide
DNA	**d**eoxyribo**n**ucleic **a**cid
DNMT1	**d**eoxyribo**n**ucleic **a**cid (DNA) **m**ethyltransferase **1**
DNMT1A	**d**eoxyribo**n**ucleic **a**cid (DNA) **m**ethyl**t**ransferase **1** **a**lpha
DNMT3a	**d**eoxyribo**n**ucleic **a**cid (DNA) **m**ethyl**t**ransferase **3** **A**lpha

DNMT3b	**d**eoxyribo**n**ucleic **a**cid (DNA) **m**ethyl**t**ransferase **3** **b**eta
dNTP	**d**eoxyribose **n**ucleoside **t**riphos**p**hates
DSC2	**d**es**m**o**c**ollin **2**
DSL	**s**traight **l**ine **d**istance
E2	**e**stradiol
eCG	**e**quine **c**horionic **g**onadotropin hormone also known as **p**regnant **m**are **s**erum **g**onatotropin (**PMSG**)
EGF	**e**pidermal **g**rowth **f**actor
ERK	**e**xtracellular signal-**r**egulated **k**inase
ETC	**e**lectron **t**ransport **c**hain
Evs	**e**xtracellular **v**esicle**s**
FACS	**f**luorescence-**a**ctivated **c**ell **s**orting
FAF-BSA	**f**atty **a**cid-**f**ree **b**ovine **s**erum **a**lbumin
FBS	**f**etal **b**ovine **s**erum
FGF	**f**ibroblast **g**rowth **f**actor
FSH	**f**ollicle **s**timulating **h**ormone
Gapdh	**g**lycer**a**ldehyde 3-**p**hosphate **d**e**h**ydrogenase
GCLC	**g**lutamate-**c**ysteine **l**igase **c**atalytic subunit
GCs	**g**ranulosa **c**ell**s**
GDF9	**g**rowth **d**ifferentiation **f**actor **9**
GJ	**g**ap **j**unctions
GJA1	**g**ap **j**unction protein **a**lpha **1** also known as **c**onne**x**in **43** (**Cx43**)
GPX	**g**lutathione **p**ero**x**idase
GSH	**g**lutathione **s**ulfur-**h**ydrogen
GV	**g**erminal **v**esicle
GVBD	**g**erminal vesicle **b**reak**d**own
H_2O_2	hydrogen peroxide
HAS1/2	**h**yaluron**a**n **s**ynthase **1** and **2**
hCG	**h**uman **c**horionic **g**onatropin
HEPES	(4-(2-**h**ydroxy**e**thyl)-1-**p**iperazine**e**thane**s**ulfonic acid)
HIOMT	**h**ydroxy**i**ndole-***O***-**m**ethyl**t**ransferase
HO2•	perhydroxyl radical
HOCl	hypochlorous acid
HSPB1	**h**eat **s**hock **p**rotein family **B** (small) member 1
ICM	**i**nner **c**ell **m**ass
IFNT	**i**nter**f**ero**n**-**τ**
IGF	**i**nsulin-like **g**rowth **f**actor
IGF1	**i**nsulin-like **g**rowth **f**actor-**1**
IVC	*in vitro* **c**ulture
IVF	*in vitro* **f**ertilization
IVM	*in vitro* **m**aturation
IVP	*in vitro* embryo **p**roduction
K^+	potasium

K-RAS	**k**irsten **ra**t **s**arcoma viral oncogene homolog
KSOM	potassium (**k**) **s**implex **o**ptimization **m**edium
LH	**l**uteinizing **h**ormone
LIN	**lin**earity
LOS	**l**arge **o**ffspring **s**yndrome
MAGUK	**m**embrane-**a**ssociated **gu**anylate **k**inase homologs
MAPK	**m**itogen-**a**ctivated **p**rotein **k**inase
MAPK13	**m**itogen-**a**ctivated **p**rotein **k**inase **13**
MARF1	**m**eiosis regulator **a**nd m**RNA** stability **f**actor **1**
Mel	melatonin
mg	**m**illi**g**ram
Mg^{2+}	**mag**nesium
MIH	**m**aturation-**i**nducing **h**ormone
MII	**m**etaphase **II**
ml	**m**illi**l**iter
mM	**m**illi**m**olar
M-MLV	**m**oloney **m**urine **l**eukemia **v**irus reverse transcriptase
MnSOD	**m**anga**n**ese **s**uper**o**xide **d**ismutase
MOET	**m**ultiple **o**vulation and **e**mbryo **t**ransfer
mOsm	milli**osm**ole
MPF	**m**aturation **p**romoting **f**actor
MPTP	**m**itochondrial **p**ermeability **t**ransition **p**ore
mRNA	**m**essenger **r**ibo**n**ucleic **a**cid
MSH	**m**elanocyte-**s**timulating **h**ormone
MT1	**m**ela**t**onin receptor type **1**
MT2	**m**ela**t**onin receptor type **2**
MT3	melatonin binding site, **m**ela**t**onin receptor subtype **3**
Na+	sodium
NANOG	**Nanog** Homeobox
NAT	serotonin ***N*-a**cetyl **t**ransferase
nM	**n**ano**m**olar
NO•	**n**itric **o**xide
O_2	oxygen
$O2^{\bullet-}$	superoxide anion
OF	**o**viductal **f**luid
$ONOO^-$	peroxynitrite anion
OPU	**o**vum **p**ick **u**p, also known as ultrasound-guided follicular aspiration
OS	**o**xidative **s**tress
$^{\bullet}$OH/HO•	**h**ydr**o**xyl radical
$^1O_2$	singlet **o**xygen
p38 α/β MAPK	**p38** MAP Kinase (**MAPK**), also called RK or CSBP (Cytokinin Specific Binding Protein)

P53	Tumor **p**rotein **53**
PBS	**p**hosphate-**b**uffered **s**aline
PETP 1/2	**pe**ptide **t**ransporter-1 and **p**eptide transporter **1** and **2**
pH	**p**otential or power of **h**ydrogen
PI	**p**ropidium **i**odide
PN	**p**ro**n**uclei
PNA	**p**ea**n**ut **a**gglutinin
PO_4^{2-}	phospahte
POU5F1	**POU** domain, class **5**, transcription **f**actor **1**
PTGS2	**p**ros**ta**g**l**andin-endoperoxide **s**ynthase **2**
PTX3	**p**en**t**ra**x**in **3**
PVA	**p**oly (**v**inyl **a**lcohol)
RNAsin	**r**ibo**n**uclea**s**e **i**nhibitor
RNS	**r**eactive **n**itrogen **s**pecies
RORα	**RAR-**related **o**rphan **r**eceptor **a**lpha, also known as NR1F1 (nuclear receptor subfamily 1, group F, member 1)
ROS	**r**eactive **o**xygen **s**pecies
RSS	**r**eactive **su**lfur **s**pecies
RT qPCR	**r**everse **t**ranscription **q**uantitative **p**olymerase **c**hain **r**eaction
SAS	**s**tatistical **a**nalysis **s**ystem
SCNT	somatic cell nuclear transfer
SIRT3	**s**i**rt**uin-**3**, mitochondria NAD-dependent deacetylase
SLC1A1	**s**o**l**ute **c**arrier family **1** member **1**
SLC2A1	**s**o**l**ute **c**arrier family **2** member **1**
SNAT	**s**erotonin ***N*-a**cetyl**t**ransferase
SO_4^{2-}	sulfate
SOD	**s**uper**o**xide **d**ismutase
SOF	**s**ynthetic **o**viductal **f**luid
SPs	**s**ubpo**p**ulation**s**
STR	**str**aightness
TALP	**ty**rode's medium base, **a**lbumin, **l**actate, and **p**yruvate
Taq Polymerase	***Thermus aquaticus*** DNA **p**olymerase
TCM199	**t**issue **c**ulture **m**edium **199**
TE	**t**roph**e**ctoderm or trophoblast
TGF-β	**t**ransforming **g**rowth **f**actor **b**eta
TJ	**t**ight **j**unctions
TPH	**t**rypto**p**han **h**ydroxylase
TRIS	**tris**(hydroxymethyl)aminomethane
UCP2	**u**n**c**oupling **p**rotein-**2**
UF	**u**terine **f**luid
UV	**u**ltra**v**iolet light
VAP	**a**verage **p**ath **v**elocity
VCL	**c**urvi**l**inear **v**elocity
VSL	**s**traight **l**ine **v**elocity

WOB	**wob**ble
ZO	**z**onula **o**ccludens
ZP	**z**ona **p**ellucida
ZP2	**z**ona **p**ellucida glycoprotein **2**
Δψm	mitochondrial membrane potential
5-MT	**5m**ethoxy**t**riptamine

An experimental study to evaluate the role of melatonin in bovine gametes competence and preimplantation embryo development *in vitro*

Juan Carlos Gutiérrez Añez

Summary

Melatonin is a potent free radical scavenger and is thought to have anti-oxidative functions in male and female gametes and early mammalian embryo development, thereby protecting gametes and early embryos from ROS (reactive oxygen species) mediated damage during *in vitro* embryo production. To determine the effects of melatonin on the developmental competence of sperm, oocytes and early embryos, we performed three major experiments using the bovine model. In the first experiment, we investigated the effects of melatonin on post-thaw sperm quality, fertilizing ability, and embryo development *in vitro*. Frozen-thawed bovine spermatozoa were incubated in the presence or absence of melatonin (0.01 nM). Sperm quality was assessed via Computer-Assisted Sperm Analysis (CASA) and flow cytometric evaluation. To determine embryo development of melatonin-treated sperm, COCs harvested from slaughterhouse derived ovaries were fertilized *in vitro* (IVF) and cultured to blastocysts. The blastocysts were analyzed for mRNA expression of a panel of developmentally important genes, including *CDH1*, *CDX2*, *MAPK13*, water transport regulator *AQP3*, apoptosis regulators *BAX* and *HSPB1*, and the pluripotency transcripts *POU5F1* and *NANOG*. Data from CASA and flow cytometry analysis did not reveal significantly improved sperm quality compared to controls. However, *in vitro* matured COCs fertilized with spermatozoa preincubated with melatonin, showed higher monospermic fertilization rates, reduced polyspermy, and enhanced embryonic development. Moreover, melatonin increased the mRNA relative abundance of *MAPK13* in the *in*

vitro-derived blastocysts, suggesting that the stimulatory effects of melatonin could be mediated via the MAPK pathway. In the second experiment, we investigated the effects of melatonin on cumulus-oocyte complexes (COCs) competence and embryo development *in vitro* in prepubertal and adult dairy cattle. Fifteen Holstein Friesian calves and fifteen adult cows underwent two rounds of transvaginal ultrasound guided ovum pick up (OPU), twice a week during a four weeks period. Harvested COCs were matured *in vitro* (IVM) with or without melatonin (0.01 nM). After IVM, COCs were fertilized (IVF) and cultured *in vitro* (IVC) to the blastocyst stage. Embryo development was assessed by determining cleavage rates, and the number of blastocysts and advanced blastocysts. Embryo quality was assessed via differential staining to determine the number of total embryonic cells and allocation to inner cell mass (ICM) or trophectoderm (TE) cells. Melatonin enhanced embryo developmental rates and increased the number of ICM and total embryonic cells in both, embryos derived from prepubertal and adult donors, indicating that supplementation of culture media with melatonin promotes embryo development in both prepubertal and adult female cows. In the third experiment, we investigated the effects of media supplementation with melatonin (1.0 nM and 0.01 nM) during IVM, IVF, and IVC on *in vitro* embryo development of COCs collected from slaughterhouse derived ovaries. The developmental potential was assessed by determining the number of morulae, blastocysts, and advanced blastocysts on days 6, 7, 7.5, respectively. The quality of the embryos was determined by counting number and type of embryonic cells (ICM or TE) and analyzing a panel of marker genes, indicative for developmental competence (*CDH1*, *CDX2*, *MAPK13*), and ion exchange and water transport (*AQP3*, *ATP1A1*, *ATP1B1*). Melatonin significantly improved embryo developmental rates, increased the number of embryonic cells, specifically TE cells, and upregulated mRNA expression of

CDH1 and *AQP3*, suggesting that melatonin is critically involved in blastocyst formation and development. Collectively, these results indicate that melatonin can protect female and male gametes and early embryos from ROS mediated damage which in turn may improve the developmental competence of gametes and early embryos from both adult and prepubertal donors. It has yet to be demonstrated whether this effect is also effective *in vivo*, i.e., after transferring the embryos to recipient animals.

Experimentelle *in vitro* Studie zur Bedeutung von Melatonin für die Entwicklungskompetenz boviner Gameten und früher Embryonalstadien

Juan Carlos Gutiérrez Añez

Zusammenfassung

Melatonin ist ein wirkungsvoller Radikalfänger mit einer starken breiten anti-oxidativen Schutzwirkung vor Schäden, die durch Sauerstoffradikale hervorgerufen werden, und soll auch wirksam sein bei männlichen und weiblichen Gameten und frühen Embryonalstadien. In der vorliegenden Arbeit wurden in drei Experimenten die Effekte einer Melatoninzugabe auf Spermien, Eizell -und Embryonenkompetenz beim Rind untersucht. Im ersten Experiment wurde der Einfluss von Melatonin auf die Spermienqualität nach dem Auftauen, die Befruchtungsfähigkeit in IVF und die *in vitro* Entwicklung der daraus entstandenen Embryonen untersucht. Dafür wurden die Rinderspermien nach dem Auftauen entweder mit oder ohne Melatonin (0,01nM) inkubiert und deren Qualität mittels CASA (Computer-Assisted-Sperm-Analysis) und Flowzytometrie im Detail analysiert. Für die Überprüfung der Befruchtungsfähigkeit von Melatonin behandelten Spermien und der Entwicklungsfähigkeit der *in vitro* fertilisierten Embryonen wurden Rinderoozyten von Schlachthofovarien gewonnen, *in vitro* gereift und befruchtet und bis zur Blastozyste *in vitro* kultiviert. In den Blastozysten wurde die mRNA Expression entwicklungsrelevanter Gene wie CDH1, CDX2, MAPK13, Wassertransport Regulatorgen AQP3, der Apoptosis Regulatoren BAX und HSPB1 sowie der Pluripotenzgene POU5F1 und NANOG untersucht. Bei der Spermienqualität wurden keine signifikanten Unterschiede zwischen den Kontrollen und der Melatonin behandelten Gruppe beobachtet. Wenn jedoch Oozyten mit Melatonin-behandelten Spermien befruchtet wurden, waren, die Fertilisationsraten signifikant erhöht, der Anteil an Polyspermie signifikant erniedrigt und die

Entwicklungsraten in der Behandlungsgruppe signifikant erhöht, verglichen mit den Kontrollen. Zusätzlich wurde ein signifikant erhöhter Level an MAPK13 Transkripten in Blastozysten aus der Behandlungsgruppe gefunden, was einen stimulierenden Effekt von Melatonin über den MAPK Signalweg nahe legt. Im zweiten Hauptversuch wurden die Effekte von Melatonin auf die Entwicklungsfähigkeit von Kumulus-Oozyten Komplexen von adulten und präpuberalen Tieren untersucht. Dafür wurden 15 Kühe und 15 Kälber der Rasse Holstein Friesian jeweils zwei Runden einer zweimal wöchentlichen ultraschallgeleiteten Follikelpunktion (OPU) für jeweils vier Wochen unterzogen. Die gewonnenen Oozyten wurden *in vitro* mit oder ohne Melatoninzugabe (0,01nM) gereift (IVM), danach fertilisiert (IVF) und bis zum Blastozystenstadium *in vitro* kultiviert (IVC). Die embryonale Entwicklung wurde anhand der Teilungsrate, der Blastozystenrate und der Expansions- und Schlupfraten ermittelt. Die Embryonenqualität wurde ferner durch eine Double Dye Färbung, womit die Gesamtzahl der Zellen ermittelt und zwischen den Zellen der inneren Zellmasse (ICM) sowie des Trophektoderms differenziert werden kann, erfasst. Die Ergebnisse dieses Versuchs zeigten, dass die Entwicklungsraten, die Gesamtzellzahlen, besonders in der ICM nach Melatonin Zuabe signifikant erhöht waren. Die Ergebnisse zeigen, dass durch Supplementierung des Mediums mit Melatonin das embryonale Wachstum sowohl bei adulten Kühen als auch bei präpuberalen Kälbern verbessert werden kann. Im dritten Versuch wurden die Effekte einer Melatoninzugabe (1,0 nM und 0,01 nM) in allen drei Schritten der *in vitro* Produktion (IVM, IVF, IVC) untersucht. Dazu wurden Oozyten, die aus Schlachthausovarien gewonnen wurden, eingesetzt. Neben der Teilungsrate und den Entwicklungsraten von Morula bis Blastozyste (Tag 6 , 7 und 7,5 der Kultur) wurden die Zellzahlen, in ICM und Trophektoderm, ermittelt. Weiterhin wurden die Embryonen auf die Expression entwicklungsrelevanter Gene (CDH1, CDX2, MAPK13)

sowie von Genen, die für den Ionenaustauch und Wassertransport in Zellen relevant sind (AQP3, ATP1A1, ATP1B1) untersucht. Die Ergebnisse dieses Versuchs zeigten, dass durch Melatoninzugabe die Entwicklungsraten, die Zellzahlen, insbesondere im Trophektoderm sowie die Expression von CDH1 und AQP3 erhöht wurden, was auf einen spezifischen Effekt von Melatonin in der Blastozystenbildung und Entwicklung hindeutet. Zusammenfassend zeigen die Ergebnisse, dass Melatonin *in vitro* die Entwicklungsfähigkeit von männlichen und weiblichen Gameten und frühen Embryonalstadien von adulten und präpuberalen Rindern verbessern kann. Inwieweit dieser Effekt auch in vivo, d.h. nach Transfer der Embryonen auf Empfängertiere wirksam ist, bedarf weiterer Untersuchungen.

Chapter I

1 General introduction

1.1 Melatonin: chemical structure, biosynthesis, and biological functions

The tryptophan-derivate indole-amine hormone melatonin (*N*-acetyl-5-methoxytryptamine) is a molecule mainly produced and secreted during the dark hours of the night by the pinealocytes of the pineal gland and has been shown to play a role in the regulation of the circadian rhythm (Tamura et al., 2009; Tan et al., 2014). The chemical structure and a brief compound summary are provided in **Figure 1**.

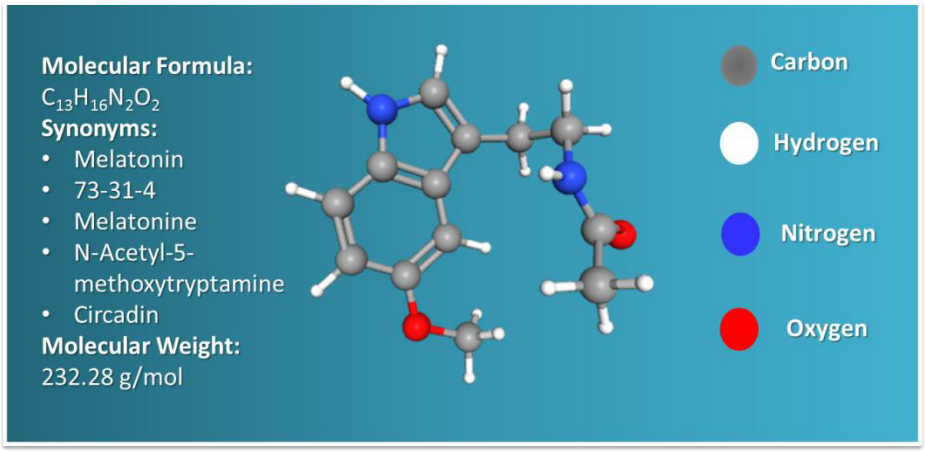

Figure 1 Chemical structure of melatonin (Adapted from PubChem: https://pubchem.ncbi.nlm.nih.gov/compound/Melatonin).

Melatonin belongs to the amino acid-derived hormones. For biosynthesis, the precursor amino acid tryptophan is taken up from the blood by the pinealocytes where it is converted into serotonin via two enzymatic reactions. First, the enzyme tryptophan hydroxylase (TPH) converts tryptophan into hydroxytryptophan, followed by

decarboxylation mediated via the enzyme aromatic L-amino acid decarboxylase (AADC), that converts hydroxytryptophan into serotonin (Zhao et al., 2019, Tan et al., 2014). In a subsequent reaction serotonin is converted into *N*-acetyl-serotonin by the enzyme arylalkylamine *N*-acetyl transferase [AANAT, in animal taxa; also referred to as serotonin *N*-acetyl transferase (NAT) in other organisms]. *N*-acetyl-serotonin is subsequently methylated to form melatonin, requiring the enzyme *N*-acetylserotonin *O*-methyltransferase (ASMT; formerly known as hydroxyindole-*O*-methyltransferase, HIOMT) (Zhao et al., 2019, Tan et al., 2014). Alternatively, serotonin can be first *O*-methylated to 5methoxytriptamine (5-MT) by ASMT; thereafter, 5-MT is *N*-acetylated by AANAT (NAT) into melatonin (Zhao et al., 2019).

Melatonin is an evolutionary very old molecule that evolved ~3.0-2.5 billion years ago (Reiter et al., 2017a). Humans know about its existence for ~100 years. Experimental evidence from studies with Rana pipiens tadpoles revealed that treatment with crude extracts of the bovine pineal gland resulted in lightening of skin pigmentation (McCord and Allen, 1917). Almost 50 years later the substance was isolated from the bovine pineal gland and structurally identified as N-acetyl-5-methoxytryptamine (Lerner et al., 1958). It was hypothesized to be a factor for lightening the skin by inhibiting melanocyte-stimulating hormone (MSH), which led to the name melatonin (Lerner et al., 1958).

Subsequently, the molecule was also discovered in several other tissues, incl., the retina (Cardinali et al., 1974), the Harderian gland (Pang et al., 1977), the gastrointestinal tract (Bubenik et al., 1980), lymphocytes (Carrillo-Vico et al., 2004), reproductive organs, such as testes (Tijmes et al., 1996) and ovaries (Itoh et al., 1997). Nowadays, accumulated evidence indicates that many mammalian tissues can synthesize functional melatonin (Reiter et al., 2013a; Reiter et al., 2013b).

Initially, melatonin was thought to regulate reproduction in photoperiodic animals (Hoffman and Reiter, 1965). However, recent work has provided solid evidence for a multifunctional role of melatonin in mammalian physiology, including circadian rhythm synchronization and sleep regulation (Zisapel, 2018; Xie et al., 2017), anti-aging (Hardeland et al., 2019; Reiter et al., 2018), inflammatory modulation (Hardeland et al., 2019, 2018), immune-modulation (Mortezaee; et al., 2019; Carrillo-Vico et a., 2013), anti-cancerogenic (Reiter et al., 2017b; Mortezaee; et al., 2019), anti-diabetic (Zhang et al., 2017), regulation of reproductive physiology (Tamura et al., 2014; Carlomagno et al., 2018), and ultimately as a very potent antioxidant in a variety of organs (Zhao et al., 2019; Reiter et al., 2017a; Tan et al., 2016; Tan et al., 2014).

1.2 The role of melatonin in the reproductive tract

Melatonin is critically involved in the circadian rhythm by secretion from the pineal gland, but local melatonin production has also been found in reproductive organs (Rocha et al., 2015; Acuña-Castroviejo et al., 2014; Tamura et al., 2013). Its accumulation in seminal plasma (Kratz and Piwowar, 2017) and follicular fluid (Tanabe et al., 2015; Tamura et al., 2013; El-Raey et al., 2011) suggests an essential role in reproductive function.

Ovarian intrafollicular melatonin levels are associated with oocyte quantity and quality and thus may serve as a biomarker of the ovarian reserve in human fertility diagnosis (Tong et al., 2017). Low melatonin concentrations in follicular fluid of infertile women were associated with severe oxidative imbalance, and supplementation with melatonin improved intrafollicular oxidative balance and oocyte quality in infertile women and ultimately resulted in increased pregnancy/birth rates (Espino et al., 2019).

Moreover, a high endogenous melatonin concentration was correlated with human sperm motility and normal sperm morphology (Ortiz et al., 2011).

Melatonin receptors have been found in reproductive organs of various species, including human preovulatory granulosa cells (Woo et al., 2001; Yie et al., 1995; Niles et al., 1999), porcine cumulus and granulosa cells (Kang et al., 2009), bovine cumulus-oocyte complexes (COCs) (El-Raey et al., 2011), sperm (Yang et al., 2014), and early embryos (Sampaio et al., 2012). The multitude of melatonin functions and the local anti-oxidative effects in reproductive organs are usually mediated via a paracrine signal, transduced by the G-protein coupled cell membrane-bound melatonin receptors type 1 and 2 (MT1 and MT2, respectively) (Slominski et al., 2012). Moreover, expression of an MT3 melatonin binding site in the bovine oocyte has been demonstrated (Sampaio et al., 2012).

The role of melatonin as an antioxidant includes direct and indirect free-radical scavenging. It involves the activity of various metabolites, such as 2-hydroxylmelatonin, 6-hydroxylmelatonin, cyclic 3-hydroxymelatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), and N^1-acetyl-5-methoxykynuramine (AMK) that possess even more active and potent scavenging functions than the melatonin molecule (Galano et al., 2013; Tan et al., 2013). Indirect antioxidant functions of melatonin include the modification of gene expression, for example, by upregulating the anti-apoptotic gene *Bcl-2* and downregulating pro-apoptotic genes such as *BAD*, *BAX*, and by enhancing the activity of other intracellular antioxidative molecules, such as superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GPx) (Bonnefont-Rousselot et al., 2010; Tamura et al., 2009). This evidence suggests that melatonin may play an essential role in reproduction by protecting gametes and early

embryos from oxidative damage, thereby ensuring viability, fertilization capacity, and early embryo development.

Figure 2 shows the multiple biological functions of melatonin in a schematic manner (Tamura et al., 2009).

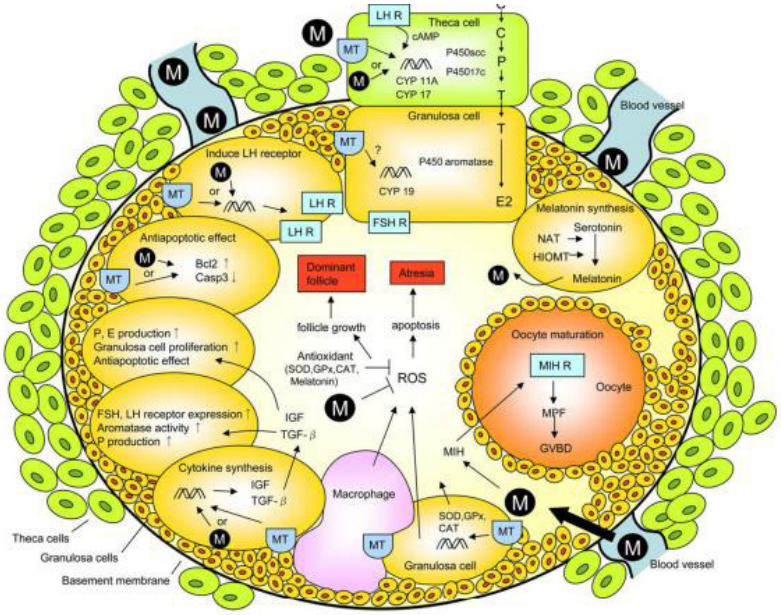

Figure 2 Schematic drawing of the presumptive roles of melatonin in the ovarian follicle (Tamura et al., 2009; Reiter et al., 2013a). Melatonin (represented in this figure M) in the follicular fluid is produced by the granulosa cells (GCs) and is also taken up from blood through the basement membrane, which affects numerous cells, especially GCs and the ovum (oocyte). Melatonin regulates the production of sex steroid hormones by regulating steroidogenic enzymatic activities or gene expression in theca cells and GCs. Melatonin also regulates LH mRNA expression, *Bcl2* and *Casp3* production, IGF and TGF-β activity, and maturation-inducing hormone (MIH). The action of melatonin is mediated via membrane receptors (MT), in particular, MT1 and MT2, and also via specific binding sites in the nucleus and cytosol. In addition to its receptor-mediated actions, melatonin also functions as a direct free radical scavenger to reduce oxidative stress at the ovary level without interaction with a receptor. Additional antioxidative functions of melatonin are mainly via indole that stimulates enzymes that detoxify free radicals. The antioxidative enzymes include superoxide dismutase (*SOD*), glutathione

peroxidase (*GPx*), and catalase (*CAT*) located in thecal cells, granulosa cells, and follicular fluid. Specifically, melatonin reduces free radical damage, particularly detrimental for the ovum, thereby maintaining its viability. The origin of melatonin in follicular fluid is twofold: from the bloodstream and local synthesis in granulosa cells. C, cholesterol; LH R, luteinizing hormone receptor; FSH R, follicle-stimulating hormone receptor; NAT, *N*-acetyltransferase; HIOMT, hydroxyindole-O-methyltransferase (currently known as acetylserotonin methyltransferase, ASMT); MIH, maturation-inducing hormone; MPF, maturation-promoting factor; GVBD, germinal vesicle breakdown; ROS, reactive oxygen species; IGF, insulin-like growth factor; TGF-β, transforming growth factor β (Source: adapted from Tamura et al., 2009; Reiter et al., 2013a).

1.3 Melatonin: an antioxidant mitochondria-targeted molecule to prevent apoptosis

Apoptosis, which refers to programmed cell death, is characterized by a complex cascade of molecular and biochemical reactions within the cells. Molecular reactions that control apoptosis and cellular fate involve various proteins (B-cell leukemia/lymphoma 2 or Bcl-2) (Antonsson et al., 2001), enzymes (caspases) (Denault et al., 2008), and other cellular compounds (cytochrome c) (Antonsson et al., 2001) produced during oxidative stress (OS).

Apoptosis in mammalian embryonic cells is often controlled by regulatory proteins from the Bcl-2 family, including pro-apoptotic and anti-apoptotic proteins that lead to permeability changes of the mitochondrial membrane (Martinou & Green, 2001). The Bcl-2 protein family plays a pivotal role in apoptotic control, including members with opposing functions (Yang and Rajamahendran, 2002). Specifically, two Bcl-2 genes, Bcl-x and Bax, have been implicated in the regulation of germ cell survival

(Rucker et al., 2000; Greenfeld et al., 2007). Bcl-2 has strong anti-apoptotic properties, while Bax is pro-apoptotic. Both genes play an essential role in regulating apoptosis in various cell types (Vogel, 2002). Members of the Bcl-2 protein family, such as the associated agonist of cell death (*BAD*) and Bcl-2 associated X protein (*BAX*), have a strong pro-apoptotic function (a pro-death protein), mainly by stimulating the release of cytochrome c from mitochondria to induce downstream caspases (proteases) that dismantle the cell (Martinou & Green, 2001).

Mitochondria are multifunctional organelles that contribute to several cellular functions, including calcium homeostasis, metabolism regulation, apoptosis, and ATP production (Tan et al., 2016). Mitochondrial damage by oxidative stress is commonly related to apoptosis and cell death. Oxidative stress induces the mitochondrial membrane to release cytochrome c, which activates the caspase system, including caspase-9 and caspase-3 (Antonsson, 2001). The trigger of caspase-3 is one of the major contributors to apoptotic processes (Denault and Salvesen, 2008; Tang et al., 2009).

During ATP production that occurs in the electron transport chain (ETC), localized within the inner membrane, some electrons inevitably leak from the ETC, and thereby reduce oxygen incompletely, which in turn leads to free radicals and other reactive oxygen species (ROS), mainly the superoxide anion ($O2^{\bullet-}$) (Tan et al., 2016). $O2^{\bullet-}$ is charged and cannot penetrate the cell membranes and remains in the mitochondrial matrix where it is detoxified by the enzyme manganese superoxide dismutase (MnSOD) into H_2O_2, a free radical with a longer half-life than $O2^{\bullet-}$ (Tan et al., 2016). H_2O_2 can be reduced to water by glutathione peroxide (*GPx*) (Burton et al., 2003). In contrast, homolysis of H_2O_2 leads to the formation of hydroxyl radical (HO•), which is a very reactive free radical triggering damage of main macromolecules such as

lipids, proteins, DNA, and carbohydrates (Tan et al., 2016). Melatonin scavenges a broad spectrum of reactive oxygen species (ROS) and reactive nitrogen species (RNS), especially HO•.

An important physiological function of melatonin is to serve mainly as a mitochondrial-targeted molecule (Tan et al., 2016), protecting the organelle from oxidative stress and the common leakage of free radicals during the respiratory chain. Melatonin can stabilize the mitochondrial inner membrane potential, regulates mitochondrial gene expression and mitochondrial permeability transition pore (MPTP) (Zhang and Zhang, 2014), which in turn optimizes mitochondrial electron transport chain (ETC) activity, and thereby decreases the leak of electrons and reduces free radical formation (Tan et al., 2016). **Figure 3** shows a model summarizing some melatonin actions in the mitochondria (Reiter et al., 2017a).

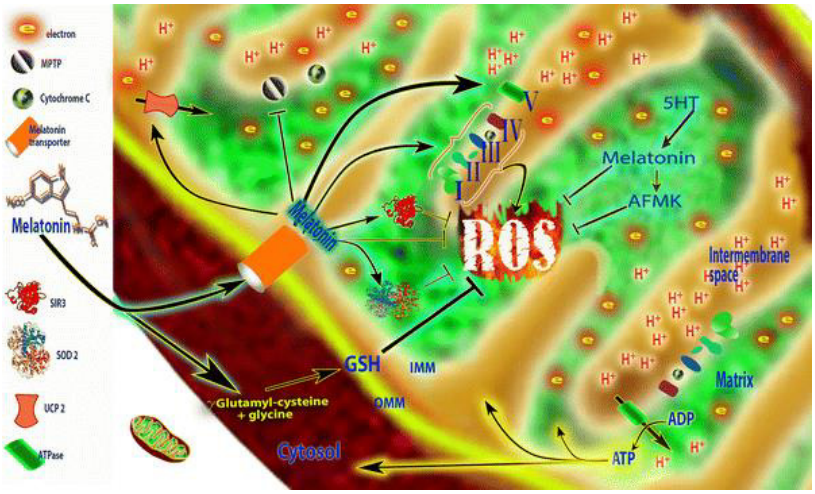

Figure 3 The targeting of melatonin to the mitochondria (Reiter et al., 2017a). Melatonin enters the mitochondria via specific transporters, PETP 1/2 (oligopeptide transporters), where it has multiple functions, including the ability to reduce oxidative damage of critical mitochondrial molecules, preservation of the functionality of these organelles and beneficial effects in case of mitochondrial malfunction. Melatonin increases the efficiency of the electron transport chain (I, II, III, and IV) and improves

ATP production (ATP-synthase). ROS produced when electrons leak from the ETC are directly scavenged by melatonin and its metabolite [*N*1-acetyl-*N*2-formyl-5-methoxykynuramine (AFMK)]. ROS are also metabolized by mitochondria superoxide dismutase (SOD2) and scavenged by glutathione (GSH) and SIRT3. Furthermore, melatonin modulates uncoupling protein (UCP2) to maintain the physiological inner mitochondrial membrane potential and prevents the opening of the mitochondrial permeability transition pore (MPTP). This limits the escape of cytochrome c when the mitochondrion is damaged by ROS. Recent evidence suggests that melatonin may also be synthesized within the mitochondria (5HT → Mel), where it is metabolized to AFMK (Source: adapted from Reiter et al., 2017a).

Melatonin is mainly synthesized by the mitochondria (Tan et al., 2016; Reiter et al., 2017a) as a self-regulatory mechanism for protecting the ancient organelle. This theory is supported by the presence of the melatonin precursor AANAT/SNAT in the mitochondria of oocytes, the observed melatonin production by isolated mitochondria, and high levels of the hormone in mitochondrial compartments (Tan et al., 2016; Reiter et al., 2017a). Current thoughts are that the primary and major function of melatonin is to serve as the first line of defense against oxidative stress and that all other functions were acquired during evolution either by adoption or as a consequence of its scavenging capacity (Tan et al., 2014; Reiter et al., 2013a). The presence of melatonin, melatonin isomers, and the enzyme serotonin *N*-acetyltransferase (SNAT) in most living organisms, including yeast, bacteria, and plants (Tan et al., 2014), is accompanied by an unchanged melatonin chemical structure throughout evolution until today (Reiter et al., 2017a). According to the endosymbiotic theory of the origin of mitochondria (Sagan et al., 1967), primitive photosynthetic bacteria (α-proteobacteria) were engulfed (phagocytosed) as food for their nutrient value by ancestral eukaryotes, developing a symbiotic association with their host (Zhao et al., 2019; Reiter et al., 2017a). Over time,

engulfed α-proteobacteria evolved into mitochondria while cyanobacteria became the chloroplast, retaining both their ability to produce melatonin (Zhao et al., 2019; Reiter et al., 2017a).

1.4 An overview of Oxidative stress and its relevance for *in vitro* embryo production

Reactive oxygen species (ROS) and Oxidative stress (OS) are frequently associated with developmental abnormalities. However, free radicals and ROS are normal compounds and by-products of cellular metabolism and show various functional activities (Lü et al., 2010). Thus, free radicals are primary and secondary messengers essential in signaling many vital cellular processes, including cell proliferation and differentiation and embryo/fetal development (Dennery, 2007; Burton et al., 2002).

Minimal redox-related signaling is required for normal progression to the blastocyst stage (Dennery, 2007). Mouse blastocysts produced high peroxide quantities within a relatively short period around the peri-hatching period compared to pre-hatching and post-hatching (hatched) blastocysts (Thomas et al., 1997). Bovine embryos require oxidative phosphorylation for ATP production at all pre-elongation development stages, where free radical production is an inevitable by-product during development (Burton et al., 2002). The relative level of OS (low, moderate, or high), or the imbalance between free radicals and ROS production and its cellular degradation (scavenging ability) can disrupt cellular homeostasis, depending on the specific stage of development (Schafer and Buettner, 2001; Agarwal et al., 2005).

Oxidative stress often triggers the increased production of oxygen-derived free radicals (ROS). Free radicals are unstable and preferentially react with other molecules due to their unpaired electrons in their atomic orbitals (Tamura et al., 2009; Lü et al.,

2010). Radicals and related non-radical species are usually derived from oxygen, nitrogen, and sulfur molecules (Lü et al., 2010), including a variety of compounds called ROS, reactive nitrogen species (RNS), and reactive sulfur species (RSS) (Lü et al., 2010; Tamura et al., 2009).

ROS and RNS are produced in various cytosolic processes, and mitochondria produce them even under normal conditions (Burton et al., 2002). Some of the ROS-derived free radicals include superoxide anion (O2•−), perhydroxyl radical (HO2•), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), singlet oxygen (1O2), hypochlorous acid (HOCl), and peroxynitrite (ONOO−), nitric oxide (NO•), whereas RNS-derived free radicals originate from nitric oxide via reaction with O2•− to form peroxynitrite anion (ONOO−). RSS-derived radicals are readily formed from thiols by reaction with ROS (Lü et al., 2010; Tamura et al., 2009). These molecules often have local toxic effects (Reiter et al., 2017a). Excessive ROS production can damage DNA, RNA, proteins, and lipids, mainly by attacking the structure of nucleic acids, amino acid chains in proteins, and double bonds in unsaturated fatty acids (Lü et al., 2010).

Oxidative stress is one of the major factors interfering with reproductive performance in cattle. It is caused by an imbalance between increased ROS production and reduced antioxidant defenses to scavenge these reactive intermediates (Belhadj Slimen et al., 2016). The excess of ROS increases the likelihood of oocytes showing cytoplasmic defects and abnormal chromosomal segregation (Belhadj Slimen et al., 2016, Roth, 2017), which in turn reduces oocyte viability and developmental competence within the follicle (Wolfenson et al., 2000).

In vivo, different oxidant defense systems (antioxidants) counteract the detrimental effects of ROS. Gametes and embryos are provided by an antioxidant

defense system that can balance OS status and neutralize ROS. This natural self-regulatory antioxidant machinery, mainly composed of superoxide dismutase (*SOD*), glutathione (*GSH*), and glutathione peroxidase (*GPx*), is essential for maintaining the developmental competence of the gametes and ensures early embryo development (Guérin et al., 2001; Wei et al., 2013).

Under *in vitro* conditions, ROS production is frequently stimulated, leading to OS that overcome the natural defense system. These supraphysiological ROS levels are caused by exposure to an oxidative environment and isolation of the oocyte from the natural protective antioxidant milieu within the follicle (Soto-Heras and Paramio, 2020). Even though cumulus-oocyte complexes (COCs) produce their own antioxidants during *in vitro* maturation (IVM), including *GSH*, *SOD*, *GPx*, and catalases (Cetica et al., 2001; de Matos et al., 2000), the excess of ROS production frequently overloads the scavenging capacity.

Increased ROS in assisted reproductive technologies can originate from endogenous (gametes, embryos) and exogenous sources (oxygen tension, light exposure, culture media, cryopreservation, and centrifugation) during *in vitro* handling (Zavareh et al., 2015). Endogenous ROS sources may originate from gametes/embryo metabolism and are stimulated by exogenous ROS, triggered by the excess of glucose in media, metallic ions, UV light, ionizing radiations, and amine oxidases (serum), and pollutants found in the *in vitro* environment (Guérin et al., 2001). The elevated presence of ROS in the *in vitro* environment can be harmful to gametes and embryos and may deteriorate the developmental competence of gametes and embryo development when occurring in assisted reproductive technologies (Zavareh et al., 2015).

1.5 Use of melatonin as an antioxidant molecule to protect gametes under *in vitro* conditions

Supplementation of the *in vitro* culture media with antioxidative compounds has emerged as a successful strategy to enhance *in vitro* embryo production (Gutiérrez-Añez et al., 2021; Soto-Heras and Paramaio, 2020; Sovernigo et al., 2017; Rocha-Frigoni et al., 2016). A multitude of natural antioxidants, incl. superoxide dismutase (Luvoni et al., 1996), glutathione peroxidase (Khalil et al., 2013), and catalase (Bain et al., 2011), and non-enzymatic antioxidants such as vitamin E (Khalil et al., 2013; Marques et al., 2010; Wongsrikeao et al., 2007), vitamin C (Sovernigo et al., 2017; Wongsrikeao et al., 2007), cysteamine (Sovernigo et al., 2017; Merton et al., 2013; Deleuze et al., 2010), carnitine (Jiang et al., 2020; Sovernigo et al., 2017), and resveratrol (Madrid Gaviria et al., 2019; Sovernigo et al., 2017; Salzano et al., 2014) have been shown to protect female and male gametes and embryos from reactive oxygen species-induced oxidative stress during *in vitro* embryo production.

Antioxidants can neutralize free radicals by accepting or donating electron(s) to eliminate the unpaired condition in disrupted molecules (Lü et al., 2010). Antioxidants can directly react with the free radicals to neutralize or destroy them; during the process, new free radicals may emerge, but these are less active than radicals that had been already neutralized (Lü et al., 2010).

Melatonin has emerged as a potent, broad-spectrum, and non-expensive free radical scavenger (Tamura et al., 2013). The antioxidative spectrum of melatonin covers both ROS and RNS (Tamura et al., 2009). Compared to classic antioxidants such as vitamin C, vitamin E, and glutathione, melatonin has a greater antioxidative capacity (Tan et al., 2013). It has been estimated that one melatonin molecule may scavenge up

to 10 free radicals, which is in sharp contrast with classic antioxidants because they typically detoxify only one radical per molecule (Tan et al., 2013). A schematic representation of the cascade reaction induced by melatonin with free radicals and its metabolites by Tan et al. (2013) is shown in **Figure 4**.

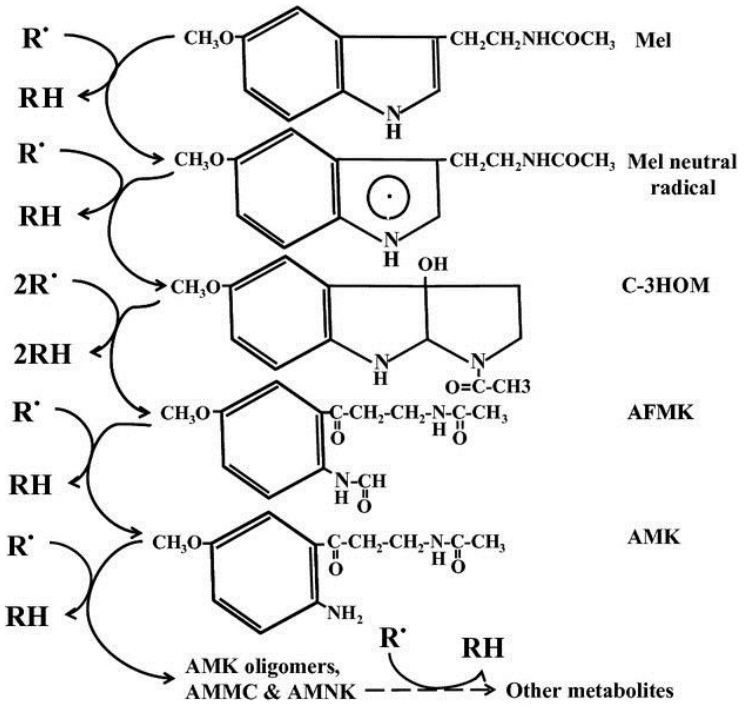

Figure 4 Cascade reaction of melatonin interaction with free radicals and its metabolites. Melatonin metabolites have a similar potency as melatonin in detoxifying ROS and reducing oxidative stress. One melatonin molecule may scavenge up to 10 free radicals, which is significantly greater than classic antioxidants, which typically detoxify one radical per molecule. R·, radical; RH, reduced agent; Mel, melatonin; C-3HOM, cyclic 3-hydroxymelatonin; AFMK, N^1-acetyl-N^2-formyl-5methoxyknuramine; AMK, *N*-acetyl-5-methoxyknuramine; AMMC, 3-acetamidomethyl-6-methoxycinnolinone; AMNK, N^1-acetyl-5-methoxy-3-nitrokynuramine; dash arrow, unidentified reactions (Source: adapted from Tan et al., 2013).

The potential use of melatonin to counteract elevated ROS production during *in vitro*-induced oxidative stress to protect the gametes against adverse ROS effects has

been studied in several species and has been shown to be able to improve the efficiency of ARTs (Tamura et al., 2014; Al-Gubory et al., 2012, 2004; Goud et al., 2008). Enhanced oocyte competence and improved *in vitro* embryo development via antioxidative effects by melatonin supplementation have been observed in humans (Zou et al., 2020; Loren et al., 2017), cattle (Marques et al., 2018; Komninou et al., 2016; Zhao et al., 2015), buffalo (Nagina et al., 2016), sheep (Tian et al., 2017), goats (Soto-Heras et al., 2018, 2019), and pigs (Yang et al., 2020; Kim et al., 2019; Wang et al., 2017).

Apoptotic effects of OS related to caspase-3/7 and the percentage of apoptotic granulosa cells can be reduced by melatonin treatment (Tamura et al., 2013). In cumulus-oocyte complexes, melatonin reduced OS-induced DNA damage, mitochondrial dysfunction, lipid peroxidation, and apoptosis of granulosa cells by decreasing free radical damage on nuclei, mitochondria, and plasma membranes (Tanabe et al., 2015).

The freezing/thawing process of spermatozoa affects plasma membrane integrity and is usually followed by a significant loss of cytoplasmic compounds with antioxidative properties (Bansal and Bilaspuri, 2010). Deleterious effects of ROS on sperm physiology are a significant risk factor leading to infertility/subfertility in males (Bansal and Bilaspuri, 2010). Melatonin supplementation protected sperm from ROS-associated damage and increased semen quality after freezing-thawing (Zhu et al., 2019). It also improved sperm cell viability, the stability of the plasma membrane, mitochondrial activity, acrosome integrity, reduced intracellular ROS levels, and enhanced development of *in vitro*-produced embryos produced from melatonin-treated spermatozoa from bovine frozen-thawed semen (Pang et al., 2016).

During bovine embryonic development, melatonin functions in various ways to reduce OS, e.g., it significantly up-regulated expression of anti-oxidative (*GPX4*, *SOD1*, *Bcl-2*) and developmentally important genes (*SLC2A1*, *DNMT1A*, and *DSC2*), while down-regulating expression of pro-apoptotic genes (*P53*, *BAX*, and *Caspase-3*) (Wang et al., 2014). Similar findings were observed in embryos derived from somatic cell nuclear transfer (SCNT), where melatonin significantly reduced apoptosis and reactive oxygen species (ROS) in bovine SCNT embryos. Furthermore, melatonin suppressed the expression of pro-apoptotic genes *P53* and *BAX* and stimulated expression of the antioxidant genes *SOD1* and *GPX4*, the anti-apoptotic gene *BCL2L1*, and the pluripotency-related gene *SOX2* in SCNT blastocysts (Su et al., 2015).

1.6 Oocyte competence: adult *vs*. prepubertal donors

In vitro embryo production (IVP) and associated technologies are now routinely used in the cattle industry. Nevertheless, several deficiencies are prevalent, including reduced oocyte competence after *in vitro* maturation, lower embryo cryotolerance, and reduced pregnancy rates compared to their *in vivo* counterparts (Ferré et al., 2020). It has been shown that approximately 90% of immature oocytes recovered from follicles at unknown stages of the estrous cycle undergo nuclear maturation *in vitro*, and about ~80% undergo fertilization following insemination and cleave at least once to the two-cell stage (Lonergan et al. 2007). Finally, only 30–40% of fertilized oocytes reach the blastocyst stage, considered to be suitable for embryo transfer. In general, these figures are still valid today (Ferré et al., 2020).

IVP with oocytes from prepubertal animals may further increase the above problems. Several studies have shown that oocytes derived from prepubertal heifers are less competent to produce embryos *in vitro* than oocytes derived from adult cows

(Palma et al., 2001; Presicce et al., 1997; Zaraza et al., 2010; Oropeza et al., 2004). Prior to puberty, most follicles that grow eventually undergo atresia, and after puberty, only a few continue to grow to the antral stage while the rest undergoes atresia; consequently, both granulosa cells and oocytes are usually characterized by a higher degree of apoptosis (Pesce and De Felici, 1994).

Numerous factors have been shown to be involved in the lower developmental capacity of oocytes derived from prepubertal donors, including deficiencies in cytoplasmic maturation (Damiani et al., 1996; Salamone et al., 2001), compromised developmental competence acquisition (Pressice et al., 1997; Camargo et al., 2005), reduced glucose metabolism and protein profiles (Steeves et al., 1999), altered mRNA profile expressions of some genes related to glucose transport (Zaraza et al., 2010; Oropeza et al., 2004), decreased mRNA levels of genes encoding steroidogenesis (Michalovic et al., 2018), altered DNA methylation profiles (Diederich et al., 2012; Bernal-Ulloa et al., 2016), and apoptosis regulation (Zaraza et al., 2010; Oropeza et al., 2004).

Increased apoptosis levels in blastocysts derived from prepubertal heifers correlate with the age of the oocyte donors (Zaraza et al., 2010). A high level of apoptosis could interfere with the acquisition of developmental competence in oocytes from prepubertal cattle. An increased proportion of apoptosis may alter connectivity and junctions within cumulus-oocyte complexes, which reduces the quality of oocytes and is often associated with poor embryonic development and low pregnancy rates (Ikeda et al., 2003).

1.7 *In vitro* embryo production (IVP)

To successfully apply IVP, it is mandatory to precisely follow a series of procedural steps to ultimately arrive at viable embryos, including the collection of gametes, maturation of oocytes, co-incubation of sperm and oocytes, and *in vitro* culture of the fertilized zygotes to the desired developmental stage. During the entire process, unique requirements regarding air, gas atmosphere, temperature, humidity, and media composition that provide the essential nutrients and elements to support gametes competence and early embryo development have to be sustained. The manual handling during *in vitro* embryo production involves essentially three major stages, 1, *in vitro* maturation (IVM), 2, *in vitro* fertilization (IVF), and 3, *in vitro* culture (IVC).

1.7.1 Cumulus-oocyte retrieval

Prior to the above steps, cumulus-oocyte complexes are to be collected either via ultrasound-guided follicular aspiration, also known as ovum pick up (OPU), or by postmortem follicular aspiration using slaughter-derived ovaries, or by ovariectomy (Ferré et al., 2020). Whatever method for follicular aspiration is used, oocytes are harvested from a very heterogeneous pool of antral follicles with 2 to 8 mm in size. Thus, follicles at different phases of follicular waves (ovulatory and non-ovulatory), dominant or subordinate (Ferré et al., 2020), including atretic ones, which cannot be morphologically or ultrasonographically discriminated from healthy oocytes, are usually collected. Kruip and Dieleman (1982) observed micromorphologically that only about 15% of the follicles present at any time of the estrous cycle were non-atretic.

1.7.1.1 Ultrasound-guided follicular aspiration (OPU)

Ultrasound-guided follicular aspiration for oocyte collection or Ovum pick up (OPU) is a technique that was initially developed for ART in humans (Gleicher et al.,

1983) and was subsequently adopted in cattle at the end of the 1980ties (Callesen et al., 1987; Pieterse et al., 1988). A major advantage of OPU is the improved exploitation of the reproductive potential of elite or genetically superior animals. OPU, in combination with IVF, has been shown to be able to improve the reproduction of superior animals compared with other biotechnologies such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET). OPU can be successfully applied in cyclic, noncyclic, pregnant cows during the first trimester of gestation, in cows that do not respond to hormonal treatments, aged animals with high genetic merit that have lost their reproductive capacity and is also a valuable tool in prepubertal cattle (Gutiérrez-Añez et al., 2021; Currin et al., 2017; Michalovic et al., 2018), thereby contributing to accelerate the dissemination of superior germplasm.

After aspirating the follicles, cumulus-oocyte complexes (COCs) are selected and graded according to morphological criteria (Bols et al. 1997 and de loos et al., 1989, 1991). COCs grade I usually are characterized by a homogeneous ooplasm with fine, dense, and uniform granulation and multiple, usually ≥ three layers of transparent and compact cumulus cells (**Figure 5**). COCs Grade II are characterized by COCs like I, but usually are a bit darker and less transparent, the ooplasm showing a more gross and dark granulation in the periphery. COCs grade III, with just a few (one or two layers) surrounding cumulus cells, or less compacted and dark cumulus rather than I and II, with dark or bright ooplasm. COCs IV are considered those with expanded or degenerated cumulus or degenerated ooplasm with noticeable dark dots that are non-suitable and usually discarded, including denuded oocytes. COCs grades I, II, and in some cases even grade III are usually considered viable and employed for IVP. Oocytes will acquire their competence for IVF and sustaining embryonic development during IVM.

Images of COCs collected from slaughterhouse-derived ovaries showing typical examples for the above quality categories adapted from Bold et al. (1997) and de loos et al. (1989, 1991) are displayed in **Figure 5**.

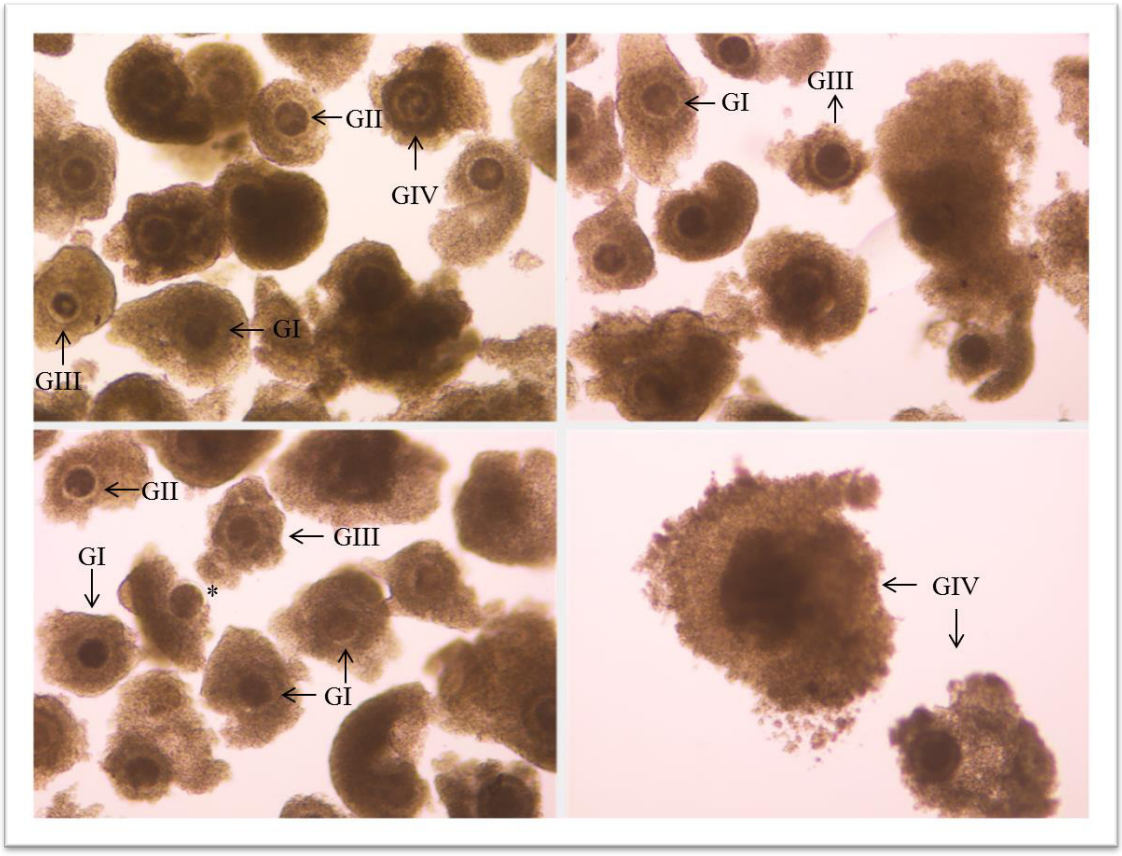

Figure 5 Bovine cumulus-oocyte complexes (COCs). Grade I (G-I): homogeneous dark ooplasm with multiple layers and compact cumulus cells (>5). Grade II (G-II): homogeneous and dark ooplasm, and at least three layers of compact cumulus cells. Grade III (G-III) dark or bright ooplasm with just a few (one or two) surrounding cumulus cells. Grade IV (G-IV): oocytes showing partially expanded and degenerated cumulus cells or degenerated ooplasm.

1.7.2 *In vitro* maturation (IVM)

Oocytes collected either via OPU or aspirated from ovaries from slaughtered animals are immature. *In vitro* maturation (IVM) is defined as the maturation of immature cumulus-oocyte complexes collected from antral follicles under *in vitro*

conditions (Edwards, 1962; De Vos et al., 2016). Usually, media employed for IVM contain gonadotropins (FSH, LH, or both) and other small molecules to promote nuclear and cytoplasmic maturation.

In vivo, the oocyte starts resumption of meiosis (maturation) within the pre-ovulatory follicle triggered by the pre-ovulatory surge of luteinizing hormone (LH) (Hyttel et al., 1989), that in turn induces progression from prophase I to metaphase II within 20-24 hours (Palma, 2008). By contrast, when the oocyte is mechanically removed from the follicular environment within the follicle, it spontaneously resumes meiosis (Edwards et al., 1962) and completes maturation *in vitro*. During the entire process, the oocyte gradually acquires developmental competence, accompanied by cellular and molecular changes that ultimately provide the gamete with the necessary machinery for completion of meiosis, to undergo monospermic fertilization, to support embryo development, and to produce normal and viable offspring (Reyes and Ross 2016).

For acquiring developmental capacity, the oocyte needs to progress from the diplotene to the metaphase II stage (nuclear maturation). The successful maturation process includes both nuclear and cytoplasmic maturation and renders the oocyte to a fertilizable state. During nuclear maturation, the nucleus, also called germinal vesicle (GV), undergoes significant structural changes, most prominent by nuclear membrane dissolution, called germinal vesicle breakdown (GVBD). The ultimate manifestation of nuclear maturation is the appearance of the pronucleus. In parallel with nuclear maturation, cytoplasmic maturation occurs, characterized by a series of changes in organelles, proteins, and transcripts that ensure the acquisition of nutrients, substrates, and mRNAs (Ferré et al., 2020). Extrusion of the first polar body is a hallmark of the first meiotic division. The oocyte then enters meiosis II and remains arrested in

metaphase II (MII) until fertilization. Under *in vitro* conditions, the time in which bovine oocytes complete maturation to MII and are ready for fertilization is 20-24 hours.

For maturation *in vitro*, cumulus-oocyte complexes are incubated under specific conditions, including well-controlled environmental requirements (38.5 °C and 5% of CO_2 in a humidified atmosphere), and a defined or semi-defined medium, usually Tissue Culture Medium (TCM199) that contains 20 common amino acids, various vitamins, and inorganic salts (Bahrami et al., 2019), supplemented with energy sources (pyruvate), hormones [gonadotropin (FSH, LH, eCG, hCG)], steroids (17β-estradiol: E2), growth factors [epidermal growth factor (EGF), Insulin-like growth factor-1 (IGF1), fibroblast growth factor (FGF)], and serum (f.ex., fetal bovine serum (FBS) or bovine synthetic albumin (BSA) (**Appendix 7.3.4**).

Concomitantly with nuclear and cytoplasmic maturation, the cumulus cells expand significantly associated with a dramatic change of the compacted shape (**Figure 6**). Cumulus expansion is a major criterion for determining the success of oocyte maturation. During expansion, cumulus cells release proteolytic enzymes that alter the polysaccharide structure of the cell bridges, which changes physical properties towards a more relaxed stage (Palma 2008).

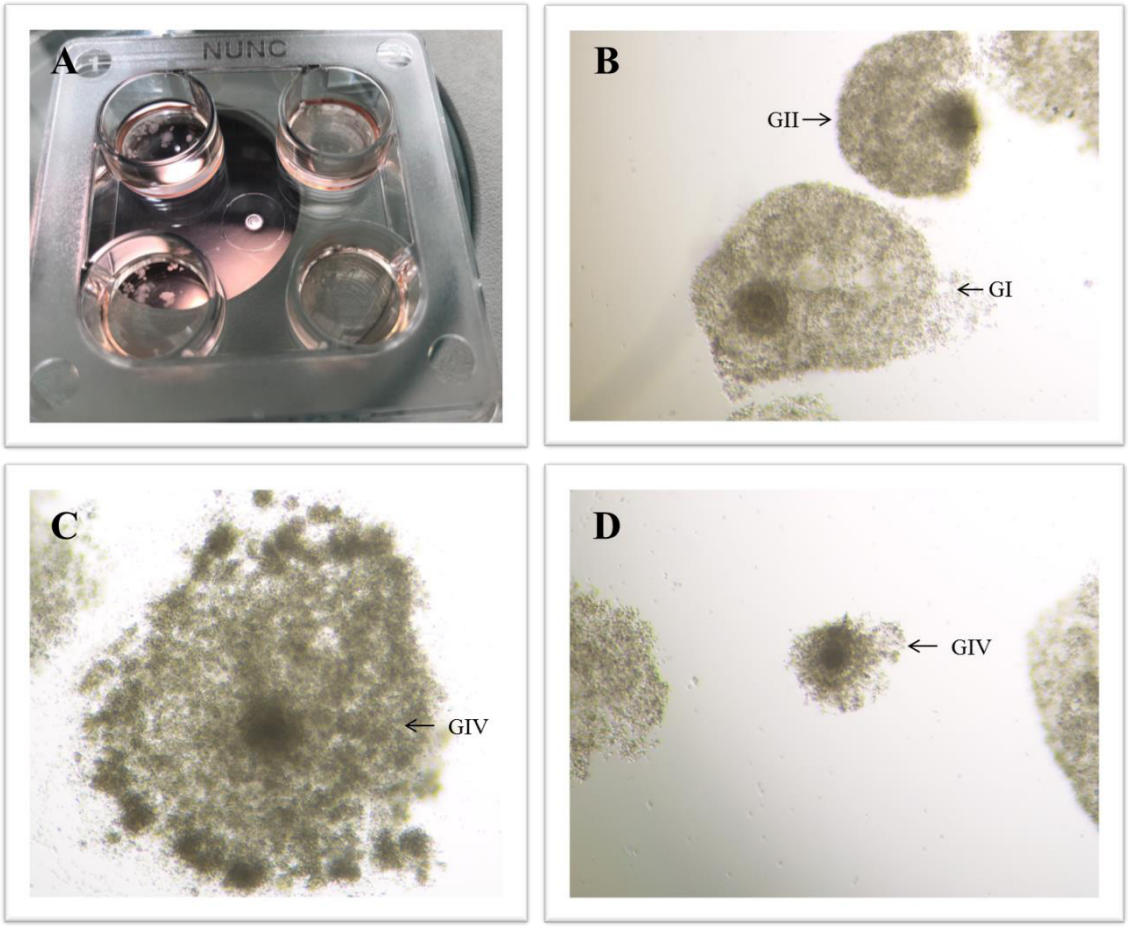

Figure 6 *In vitro* maturation. A) Four-wells dish containing expanded cumulus-oocyte complexes. Expanded COCs can be macroscopically recognized. **B)** *In vitro m*atured oocytes with expanded cumulus with different quality categories (grades I and II). **C)** *In vitro m*atured oocyte quality IV (Degenerated cumulus cells at the periphery). **D)** *In vitro m*atured oocyte quality IV (few surrounding cumulus cells).

1.7.3 *In vitro* fertilization (IVF)

After completion of maturation, COCs are co-incubated for another 18-20 hours with the sperm to undergo fertilization. For successful fertilization, the oocytes must be penetrated by a single sperm, must block the penetration of additional sperm, and must be able to form the maternal and paternal pronuclei (Parrish 2014). For fertilization, the sperm must reach their fertilizing capacity by undergoing *in vitro* the process of capacitation that triggers the acrosome reaction characterized by a series of

biochemical modifications once the sperm has reached the zona pellucida (ZP) (Parrish et al. 1986; Parrish 2014; Palma 2008).

Prior to capacitation, the spermatozoa are washed and selected to remove freezing media, seminal plasma, debris, and dead sperm, ultimately arriving at the live and motile sperm fraction (Ferré et al., 2020). Popular methods for this selection include the swim-up procedure (Parrish and Foote, 1987), density gradient centrifugation, or discontinuous density gradient via passing the sperm through a colloidal solution. Commercial products of colloidal solutions include Bovipure® and Percoll®, which constitute a colloidal silica solution. The *in vitro* fertilization medium (Fert-TALP, Parrish, et al., 1986) is based on Tyrode's medium, enriched with albumin, lactate, and pyruvate (TALP) and capacitating factors (heparin, hypotaurine, and epinephrine), bovine serum albumin fatty acid-free (BSA- FAF), and antibiotics such as gentamicin (**Appendix 7.3.5**).

The minimum number of sperm needed per oocyte can vary according to bulls, and types of semen [fresh, refrigerated, frozen (unsorted or traditional, sex-sorted)], even across IVF labs. A concentration of 100,000 sperm cells per every 100 ml medium is used in our lab with good fertilization rates. The fertilization rate, usually measured as the cleavage rate 48-72 hours post-insemination, ranges between 70-85% (Ferré et al., 2020). Other methods to determine successful fertilization include visual inspection or staining to confirm extrusion of the second polar body and pronuclei assessment (**Figure 7**).

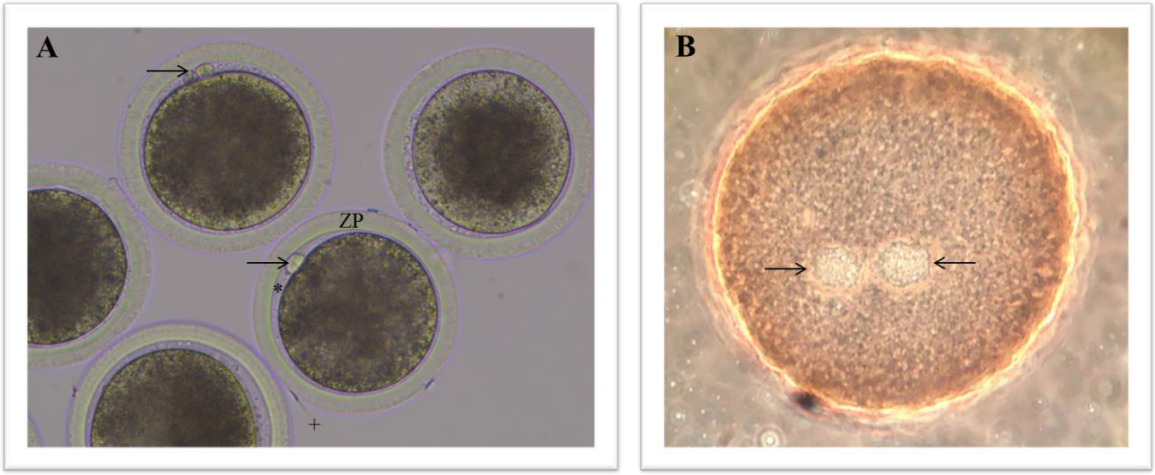

Figure 7 Bovine fertilized oocytes (= zygotes). A) Zygotes after 20 hours of *in vitro* fertilization. ZP: zona pellucida, arrows: show the second polar body. * Shows perivitelline space. + Shows a sperm attached to the ZP. B) Pronuclear (PN) determination of fertilization via Lacmoid staining to evaluate the *in vitro* fertilization success. Arrows show the two pronuclei.

1.7.4 *In vitro* culture

The goal of IVC is to support embryo development until transfer or cryopreservation. The duration of embryo culture varies amongst species; in humans, short culture periods of 3-4 days after fertilization are common, and embryo transfer can be performed in early embryonic stages (cleavage stages, early morulae), while bovine embryo development until the blastocyst stage usually lasts for 7-7.5 days after fertilization. During the IVC period, embryos must be provided with all conditions that promote the development and maintain developmental competency.

Common conditions include a temperature of 38.5 °C, a humidified atmosphere, an osmolality of 270-300 mOsm, 5 % O_2 and CO_2, and a basic culture medium that is able to mimic the *in vivo* conditions as much as possible. All *in vitro* embryo culture media contain salts, mostly six inorganic ions: Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}, and SO_4^{2-}, and

also PO_4^{2-} (Baltz, 2012), and are supplemented with amino acids, pyruvate, and bovine serum albumin fatty acid-free (BSA-FAF), or fetal bovine serum (FBS) (**Appendix 7.3.6**). The use of serum has been shown to increase the incidence of large offspring syndrome (LOS) in bovine and should be replaced by inert molecules (Lazzari et al., 2002).

The three most frequently employed media include Synthetic Oviductal Fluid supplemented with amino acids (SOFaa) (Tervit et al., 1972), potassium simplex optimization medium with amino acids (KSOMaa) (Erbach et al., 1994), and Charles Rosenkrans one amino acids (CR1) medium (Rosenkrans and First, 1994: reviewed in Palma 2008).

Eighteen to twenty hours post-fertilization (hpi), cumulus cells from presumptive zygotes are removed mechanically by pipetting or vortexing the oocytes in tissue culture medium (TCM) 199 medium with HEPES modification. They are then placed into drops of small volume (30-50 µL) or into four-well dishes containing 500 µL *in vitro* culture (IVC) medium based on synthetic oviductal fluid [SOF, described in Tervit et al. (1972) and Gandhi et al., 2000)], enriched with 4 mg/ml of BSA-FAF covered with 600 µL mineral oil already incubated at 38.5 °C, with 5% of CO_2 and 5% of O_2 in a humidified atmosphere to maintain osmolality and ionic composition, until blastocyst formation (day 7-7.5) (**Figure 8**). Usually, 48-72 hours after fertilization, cleavage rate is determined, and non-fertilized oocytes are removed. This practical routine is common in many commercial IVF labs. The culture system could be either oil-free or covered with mineral oil.

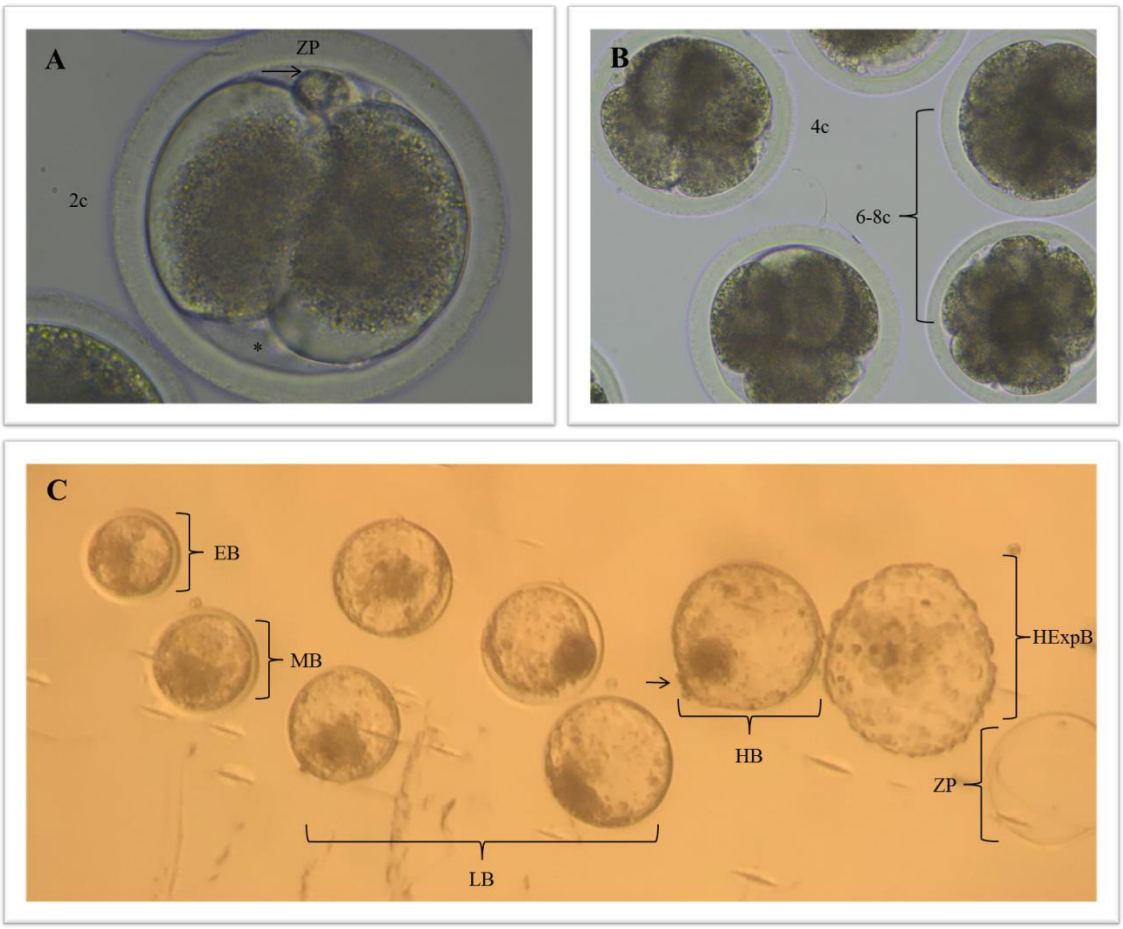

Figure 8 Bovine *in vitro*-derived embryos at different stages of development after IVF. A and B) Embryos at days 2 and 3, respectively (cleavage rate assessment). A) 2c: two cells, ZP: zona pellucida, arrows: show the second polar body, * Shows perivitelline space. B) 4c: four cells, 6-8c: six to eight cells. C): Blastocysts at day 7.5 (Blastocyst rate assessment): EB: early blastocyst, MB: mid blastocyst, LB: Late blastocyst (expanded blastocyst), HB: hatching blastocyst (arrow indicate where the ZP is breaking), HExp: Hatched and expanded blastocyst, ZP: zona pellucida empty. LB, HB, and HExp are advanced stages of embryo development.

1.8 Overview of cell lineage specification during bovine preimplantation embryo development

Preimplantation embryo development comprises a series of cellular and molecular events spanning the time from monospermic fertilization to embryo implantation. During this period, the embryo develops freely in the oviducts (until day

3-5 depending on species) and the uterus (days 6-12 depending on species) and undergoes continuous cell divisions (cleavage), compaction to form the morula and the cavitation with the formation of two major cell compartments (ICM and TE) and the blastocoel in the blastocyst, finally hatching from the zona pellucida followed by attachment to the uterine wall. Up to the morula, the bovine embryo does not grow in size (Bó and Mapletoft 2013), remains at ~150 μm in diameter; size increases from the late blastocyst (day 8) onwards in a species-specific pattern, for instance, in the bovine, the blastocyst, still within the zona pellucida, continues to expand by 1.5 times to reach a diameter of about 200 μm. Later, between 9 and 10 days old, expanded blastocysts hatch from the zona pellucida, and hatched blastocysts undergo further expansion before they start to elongate approximately 13 days after fertilization (Morris et al., 2001).

As the embryo develops, two essential processes, compaction, and cavitation occur in a well-orchestrated and synchronized manner, which gives rise to the transformation of the compacted morula to the blastocyst stage (Gordon, 2003). In the early morula stage (8-16 cells), all cells (blastomeres) have the same round shape and a similar size, have soft junctions to each other, and their boundaries can be easily identified under the microscope. At the compacted morula stage, the outer blastomeres start forming tight junctions, become flattened, and cannot be distinguished from each other (Negrón-Pérez, 2017). Compaction, with the formation of tight junctions, is the first morphogenetic event during preimplantation embryo development in the late morula (Watson et al., 2004) and involves the acquisition of polarity in the outer embryonic cells, with the formation of tight junctions (Gordon, 2003; Watson et al., 2004). Tight junctions are formed when the embryo has around 32-64 cells and is associated with a reduction of the total volume of the embryo, obscuring the individual cell boundaries (Negrón-Pérez, 2017).

Cavitation or blastocoel formation leads to the fluid-filled cavity within the blastocyst. This phenomenon gives rise to the segregation of two different cell types. One group of cells is aligned parallel to the zona pellucida, taking over a primary role as transporting epithelium, fundamental for ion and water transport (Watson et al., 2004; Gordon, 2003). This specialized group of cells is called the trophectoderm or trophoblast (TE). Another group of cells forms a clump and is tightly packed, and is termed as the inner cell mass (ICM), or embryoblast. The two cell types (TE and ICM) can be recognized in the blastocyst around day 7 of development. **Figure 9** shows a schematic representation of the blastocyst with the two cell types (TE and ICM) and the fluid-filled cavity (blastocoel).

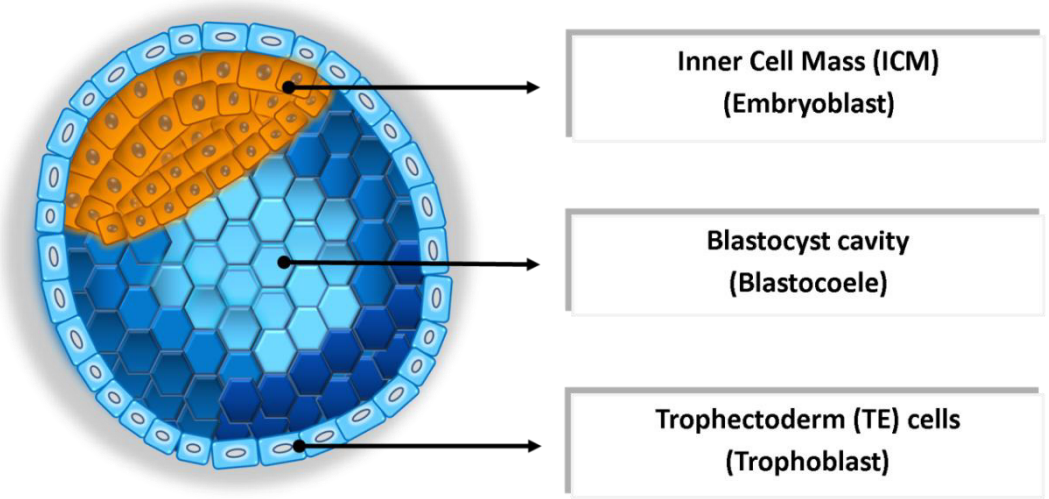

Figure 9 Schematic representation of a blastocyst. Two cell lineages are differentiated: trophectoderm (TE) and inner cell mass (ICM), and the fluid-filled cavity (blastocoel).

Prior to implantation, a second cell differentiation event (between days 7 and 9) results in the partition of the ICM and gives rise to the pluripotent epiblast (Gordon, 2003) and the differentiated hypoblast (Negrón-Pérez et al., 2018). At this stage, three

cell layers give rise to the fetal tissues (epiblast), the yolk sac, and extraembryonic endoderm (hypoblast), and the placental tissues (TE) (Morris et al. 2012). For implantation, the TE cells attach to the endometrium, and a species-specific embryonic/fetal growth and developmental pattern ultimately leads to a fully grown fetus. The ICM gives rise to the embryo proper with its three specialized germ layers (endoderm, mesoderm, and ectoderm).

This preimplantation program could be defined as the 'de novo' epithelium differentiation (Watson and Barcroft (2001). Essential genes that control development include families that encode for cell polarity, cell junctions [adherens junctions (AJ, anchoring junctions), tight junctions (TJ, impermeable junctions), gap junctions (GJ, communicating junctions)], cytoskeleton formation, water channel, and both ion transport and exchange (Bell et al., 2008; Watson and Barcroft, 2001).

The formation of adherens junctions consisting of E-cadherin and catenin complexes is essential for compaction (Bell et al., 2008). E-cadherin (a cell-adhesion molecule) is a member of the cadherin superfamily found in epithelial cells (Watson et al. 2004) and is one of the first compaction-associated molecules (Bell et al., 2008). Other molecules that form the complex heteromeric structure of AJs include cytoplasmic proteins, called catenins, that bind to cytoskeletal components, such as actin and filament microtubules (Meng & Takeiki 2009). The AJs are essential for cell adhesion and precede the establishment of other cell-to-cell contacts, such as desmosomes and tight junctions (TJ) (Watson et al. 2004). These molecular complexes interact with other proteins, including signaling molecules, making the AJs highly dynamic structures (Meng & Takeiki 2009).

Other proteins involved in compaction include the zonular tight junction (TJ) that constitutes scaffolding proteins belonging to the MAGUK (membrane-associated guanylate kinase homologs) family, also known as the Zonula occludens or ZO proteins, ZO-1 and ZO-2 (Sheth et al., 2008). Embryos lacking the ZO-2 protein exhibited delayed blastocoel formation and had an increased assembly of ZO-1, suggesting ZO protein redundancy as a compensatory mechanism (Sheth et al., 2008). In contrast, a ZO-1 knockdown, or combined ZO-1 and ZO-2 knockdown, was associated with severe blastocoel formation inhibition, indicating distinct roles for ZO proteins in blastocyst morphogenesis (Sheth et al., 2008).

Mitogen-activated protein kinase (MAPK) pathways mediate critical cellular processes in mouse preimplantation development (Natale et al. 2004). Cellular proliferation and differentiation and development are regulated by several pathways, with the MAPK signaling cascade playing an essential role herein (Aroor & Shukla 2004). Active p38 α/β MAPK signaling is required for development from the 8- to 16-cell stage to the blastocyst stage, regulating filamentous actin during murine preimplantation development (Natale et al. 2004). Furthermore, it is also necessary for blastocyst formation in the mouse (Maekawa et al. 2005). In contrast, early bovine embryos do not exclusively rely on p38 MAPK activity to complete development to the blastocyst stage (Madan et al., 2007). Both MAPK signaling and the extracellular signal-regulated kinase (ERK) pathway are required for bovine blastocyst formation (Madan et al. 2005).

For blastocyst expansion, a differentiated polarized epithelium is formed to sustain water transport for fluid accumulation during cavitation (Gordon, 2003). Water may move across the epithelium by (1) diffusion; (2) as a by-product of co-transporter

activity; or (3) via Aquaporins (AQP) or water channels (Watson et al. 2004; Barcroft et al., 2003). Aquaporins (molecular water channels) are expressed during early bovine embryo development and ultimately are essential for blastocyst formation (Sponchiado et al., 2017). AQP-mediated water movement across the trophectoderm, coupled with the establishment of a tight junction to block water leakage, is the primary mechanism that promotes blastocyst formation (Watson et al. 2004).

The fluid transfer across the outer blastomeres is critically regulated by modulation of Na+-K+-adenosine triphosphatase (ATPase) activity. The molecule is located in the basolateral membrane of the TE cells (Gordon, 2003; Houghton et al., 2003). Na/K-ATPase isoforms, such as the Na/K-ATPase β1 subunit and α1β1 isozyme (MacPhee et al. 2000), are also required for regular assembly of tight junction-associated proteins (Madan et al. 2005; Violette et al., 2006). The γ -subunit of the Na-K-ATPase modulates the Na+-pump activity of the TE during the expansion of the bovine blastocyst (Barcroft et al. 2002). Houghton et al. (2003) found a transient but significant increase of sodium pump activity in bovine and human expanding blastocysts. Approximately 60 and 36% of the ATP produced is used by the sodium pump during blastocoel expansion in human and bovine blastocysts, respectively (Houghton et al. 2003). Similarly, Barcroft et al. (2002) reported that the Na-K-ATPase γ-subunit modulates Na+-pump activity in both apical and basolateral areas of the TE during formation and expansion of the bovine blastocyst.

Concomitantly with blastocyst expansion, the zona pellucida is increasingly becoming thinner and ultimately is ruptured and allows the embryo to escape. This process is defined as hatching. It commonly occurs 7 to 9 days post fertilization in bovine embryos (Betteridge and Flechon 1988). After hatching from the zona pellucida,

the embryo increases embryo-maternal communication via intimate contact between TE and endometrium culminating in implantation, maternal recognition, and pregnancy establishment.

Negrón-Pérez et al. (2017) described three mechanisms involved in embryo hatching: 1. mechanical forces exerted on the zona pellucida as the blastocyst expands; 2. softening of the zona pellucida by enzymatic degradation, and 3. penetration of the zona pellucida by projections of trophectodermal cells. Enzymes involved in the hatching process include Trypsin-like protease (Sawada et al., 1990), other proteases (Mishra et al., 2000), and urokinase-type plasmogen activator (Berg et al., 1992). Additionally, blastocyst expansion contributes to the zona pellucida weakness by reducing it to one-fifth (1/5) of its original size.

1.9 Embryo competence: *in vitro vs. in vivo*-derived embryos

In vitro-derived embryos are frequently less competent than their *in vivo*-derived counterparts, mainly reflected in the lower pregnancy rates (~10% to 40%), with ~60% of these pregnancies failing during the first six weeks of gestation, clearly indicating reduced competence of IVP derived embryos (Ealy et al., 2019). Important features involved in the reduced competence in *in vitro*-derived embryos include a reduced number of embryonic cells, a less elongated conceptus, a smaller embryonic disk, compromised yolk sac development, compromised development of binucleate cells, and cotyledons, and reduced placenta vascularization (Ealy et al., 2019). Interestingly, the smaller embryonic disc observed in IVP-derived embryos was preferentially found in female conceptuses rather than their male counterparts (Bertolini et al., 2002). A reduced number of trophectodermal (TE) cells might be associated with an increased number of embryonic losses, while a decreased number of inner cell mass (ICM) cells is

thought to increase the risk of fetal mortality or impaired development (Carlomagno et al., 2018). Recently, we found that embryos derived from adult and prepubertal oocytes possessed an increased number of ICM, more total embryonic cells, and a higher proportion of advanced blastocysts when cultured in a medium supplemented with melatonin (Gutiérrez-Añez et al., 2021).

Oocyte developmental potential could be expressed as the percentage of oocytes that can develop to the blastocyst stage (Gandolfi et al., 1997). However, a high figure will not guarantee that the embryo will survive cryopreservation or/and develop to term after transfer to a recipient.

Previously, Overström (1996) defined embryo viability as the capability to continue its development *in vivo*. Thus, embryo viability assessment aims to identify embryo(s) with the highest chance to produce and maintain a pregnancy (Aydiner et al., 2010).

Methods for the evaluation of embryo viability mainly include five different strategies (Overström 1996): Non-invasive methods: (1), morphological quality evaluation, (2), assessment of *in vitro* developmental capacity (blastocyst development), (3), metabolic test, and Invasive methods: (4), biopsies to analyze cell samples, and (5) differential staining (ratio between inner cell mass and trophectoderm cell numbers). An example of embryonic cell counting and differential staining in a bovine blastocyst is shown in **Figure 10**.

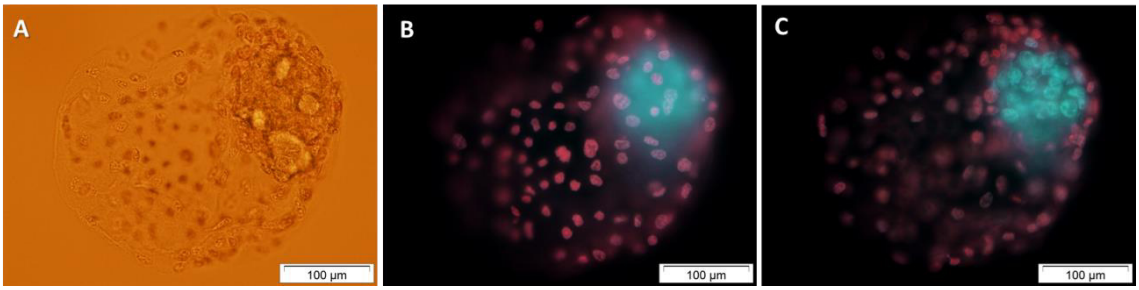

Figure 10 *In vitro*-produced hatched blastocyst (7.5 days) under the fluorescence microscope. (A) Brightfield (B and C) Differential staining images focusing on different layers of the same embryo. Bar scale= 100 µm. Embryo derived from melatonin treatment.

Metabolic tests and studies include measurement of glucose, pyruvate, lactate (Khurana and Niemann, 2000) or amino acids in embryo culture media, determination of oxygen consumption by the early embryo, and more recently, an analytical evaluation of the entire embryonic metabolome (Uyar and Seli, 2014; Aydiner et al., 2010). Other approaches for embryo viability assessment include proteomics (Katz-Jaffe et al., 2006), messenger RNA (mRNA) expression (Wrenzycki et al., 2007), and more recently, genomic and transcriptomic assessment at the single-cell level (Fuchs Weizman et al., 2019). In human ARTs, embryo development is increasingly being monitored using non-invasive time-lapse systems (Armstrong et al., 2018), incorporating automated predictors through algorithms (Dirvanauskas et al., 2019; Alegre et al., 2019).

1.10 Genes of interest in this study

Expression analysis of a panel of marker genes related to embryo developmental competency was performed in this thesis to gain insight into the effects of melatonin on bovine gametes and embryo development. The following is a brief description of the genes analyzed in the present thesis.

- **Epithelial cadherin 1 (*CDH1*)**: This protein-coding gene encodes a classical cadherin molecule of the cadherin superfamily. The cell adhesion molecule E-cadherin is one of the first compaction-associated molecules critically involved in cell lineage specification during preimplantation development (Watson et al. 2004; Bell et al., 2008). *CDH1* is essential during morula compaction (Maître et al., 2015), lineage allocation, and cell polarity during blastocyst formation (Johnson and McConnel, 2004).
- **Caudal type homeobox transcription factor 2 (*CDX2*)**: This protein-coding gene is a member of the caudal-related homeobox transcription factor gene family and is closely associated with preimplantation embryo development. The caudal-type homeobox 2 (*CDX2*) is essential for early preimplantation embryo development and is specifically involved in the differentiation of the two lineages, inner cell mass (ICM) and trophectoderm (TE). In the bovine, *CDX2* downregulation caused delayed blastocyst formation (Sakurai et al., 2016), while its mRNA depletion predisposed to loss of TE epithelial layer integrity (Goissis and Cibelli, 2014).
- **Mitogen-Activated Protein Kinase 13 (*MAPK13*)**: This protein-coding gene encodes a member of the mitogen-activated protein (MAP) kinase family. Early bovine embryos critically depend on both MAPK signaling and the extracellular signal-regulated kinase (ERK) pathway to complete development to the blastocyst stage (Madan et al. 2005).
- **Aquaporin 3 (*AQP3*)**: This protein-coding gene encodes the water channel protein aquaporin 3. Aquaporin molecules play a crucial role in cavitation (blastocyst formation) by mediating the fluid transfer across the outer

blastomeres and the trophectoderm (Watson et al., 2004; Sponchiado et al., 2016).

- **ATPase Na+/K+ Transporting Subunit Alpha 1 (*ATP1A1*)**: One of the protein-coding genes that belong to the family of P-type cation transport ATPases and the subfamily of Na+/K+ -ATPases. Na/K-ATPase alpha-subunit isoforms are found throughout early bovine embryo development and may contribute to blastocyst formation (Betts et al. 1998). Embryos that cannot express alpha1 subunits of Na/K-ATPase can form a blastocoel but fail to maintain blastocyst integrity and get lost during the peri-implantation period (Kidder et al., 2005).
- **ATPase Na+/K+ Transporting Subunit Beta 1 (*ATP1B1*)**: *ATP1B1* is one of the protein-coding genes that belong to the Na+/K+ family, and H+/K+ ATPases beta chain proteins, and the subfamily of Na+/K+ -ATPases. Madan et al. (2007) observed that the beta1 subunit of the Na/K-ATPase is required for blastocyst formation and necessary to maintain normal Na/K-ATPase distribution and localization of tight junction-associated polypeptides during preimplantation development.
- **BCL2 Associated X, Apoptosis Regulator (*BAX*)**: This gene belongs to the BCL2 protein family. *BAX* is a pro-apoptotic regulator critically involved in pro-apoptotic pathway signaling, including apoptosis or programmed cell death and apoptotic mitochondrial changes. Poor quality cumulus-oocyte complexes exhibit higher *BAX* protein levels and a reduced BCL-2/BAX protein ratio rather than good-quality COCs (Emanuelli et al., 2019).
- **Heat Shock Protein Family B (Small) Member 1 (*HSPB1*)**: This gene encodes HSP20, a member of the small heat shock protein family, involved in

response to environmental stress. It is involved in the negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway. Increased protein abundance of *HSPB1* is found until the zygotic stage, followed by a decrease again until the 4-cell stage, suggesting a role in fertilization and zygote formation (Deutsch et al., 2014).

- **POU domain, class 5, transcription factor 1 (*POU5F1*)**: This gene encodes a transcription factor containing a POU homeodomain that plays a critical role in early embryonic development and stem cell pluripotency. Targeted disruption of the bovine *POU5F1* locus prevented blastocyst formation and induced *CDX2* aberrant expression (Daigneault et al., 2018). Simmet et al. (2018) observed that *POU5F1* is required for NANOG expression in bovine blastocysts.
- **Nanog Homeobox (*NANOG*)**: The protein encoded by this gene is a DNA binding homeobox transcription factor involved in embryonic stem (ES) cell proliferation and pluripotency. Recent research by Ortega et al. (2020) showed that deletion of NANOG does not interfere with segregation or maintenance of the TE. Instead, it was required to derive and maintain the pluripotent epiblast in the bovine embryo.

1.11 Goal and relevance of the current investigation

The limited developmental potential and lower pregnancy rates after the transfer of IVP-derived embryos compared to their *in vivo* counterparts remain a major challenge for an efficient application of modern ARTs in the cattle industry.

One of the many factors contributing to the limited development has been identified as oxidative damage, mainly via enhanced ROS formation. Several investigations have shown that supplementation of the culture media with melatonin

improves gamete viability and *in vitro* embryo production (IVP). The beneficial effects of melatonin have mainly been associated with the antioxidative function of the molecule. However, an in-depth understanding of its functionality during early embryo development is lacking. The goal of the present dissertation was to expand current knowledge on the role of melatonin during bovine early development and to gain a better understanding of the underlying biological mechanisms of melatonin in supporting gametes viability, embryo development and competence *in vitro*.

The goal of the first experiment (manuscript no.1) was to evaluate the role of melatonin on sperm functionality using an established bovine IVF-protocol by assessing plasma membrane and acrosome integrity, mitochondrial activity, and sperm kinetics, followed by *in vitro* fertilization and early embryo development *in vitro*. To gain insight into the underlying mechanisms of the melatonin action, we analyzed a panel of marker genes related to important developmental pathways, i.e., lineage allocation and cell polarity Cadherin 1 (*CDH1*), the caudal type homeobox 2 (*CDX2*), the regulator of cell proliferation and differentiation MAP kinase activity (*MAPK13*), the water transport gene aquaporin 3 (*AQP3*), two apoptosis regulators (BCL2 Associated X, Apoptosis Regulator (*BAX*), and Heat shock protein beta-1 (*HSPB1*), and pluripotency transcripts [POU domain, class 5, transcription factor 1 (*POU5F1*), and Nanog Homeobox (*NANOG*)]. Results showed that incorporating melatonin into the IVF sperm-preparation protocol increased sperm fertilization ability, reduced polyspermy rate, and enhanced early embryonic development *in vitro*. This report shows for the first time that treatment of sperm after freezing and thawing with melatonin affected mRNA expression of *MAPK13* in *in vitro*-derived blastocysts suggesting a long-term effect of melatonin via the paternal gamete on embryonic development.

The next experiment (manuscript no.2) evaluated the effects of melatonin supplementation during *in vitro* maturation on the developmental competence of oocytes isolated from both prepubertal and adult donors and embryo development *in vitro* after IVF. Results provide the first scientific evidence that melatonin can improve bovine prepubertal oocyte competence *in vitro*. Increased embryonic cell counts and a higher allocation of cells to the inner cell mass in adult and prepubertal donors in the melatonin group indicated improved blastocyst quality. The improved embryo quality by melatonin could be used to increase the efficiency of IVP systems under field conditions as it is relatively easy to incorporate melatonin into a functional IVP system.

The last experiment (manuscript no.3) investigated the effects of melatonin on the developmental capacity of oocytes and embryo competence *in vitro*, mainly by looking at the mRNA expression of two developmentally-important gene families, known to be critically involved in early preimplantation embryo development, incl. (I) genes involved in morula and blastocyst formation, i.e., type 1 epithelial cadherin 1 (*CDH1*), the caudal type homeobox 2 (*CDX2*), and the regulator of MAP kinase activity (*MAPK13*), and (II) genes involved in ion exchange and water transport genes such as aquaporin 3 (*AQP3*), the ATPase Na+/K+ transporting subunits alpha 1 (*ATP1A1*), and beta 1 (*ATP1B1*). The data indicated that *CDH1* and the ion exchange and water transport gene *AQP3* are critically involved in melatonin-mediated regulation of oocyte competence and embryo development *in vitro*. We also report for the first time that the use of ethanol as a vehicle for melatonin could affect mRNA-expression of important developmental genes encoding *ATP1A1*, *CDH1*, *CDX2*, and *MAPK13*, suggesting that alternative less toxic vehicles for dissolving melatonin should be considered.

Chapter II

2 Manuscript 1

Melatonin improves rate of monospermic fertilization and early embryo development in a bovine IVF system

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JCGA design the experiment, performed the experimental trials, analyzed data, and drafted the manuscript.

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Abstract

The developmental competence of male and female gametes is frequently reduced under *in vitro* conditions, mainly due to oxidative stress during handling. The amino-acid derived hormone melatonin has emerged as a potent non-enzymatic antioxidant in many biological systems. The goal of the present study was to evaluate the effects of melatonin on post-thaw sperm quality, fertilizing ability, and embryo development and competence *in vitro* after *in vitro* fertilization. Frozen-thawed bovine spermatozoa were incubated either in the presence of 0.01 nM melatonin (MT), or its solvent (ethanol; Sham-Control), or plain Tyrode's Albumin Lactate Pyruvate medium (TALP, Control). Computer-Assisted Sperm Analysis (CASA) and flow cytometry data after 30 min, 120 min, and 180 min incubation did not reveal any significant effects of melatonin on average motility parameters, sperm subpopulation structure as determined by hierarchical cluster, or on the percentage of viable, acrosome intact sperm, or viable sperm with active mitochondria. Nevertheless, *in vitro* matured cumulus-oocyte-complexes fertilized with spermatozoa which had been preincubated with 0.01 nM melatonin (MT-Sperm) showed higher ($P < 0.01$) rates of monospermic fertilization, reduced ($P < 0.05$) polyspermy and enhanced ($P < 0.05$) embryo development compared to the Control group. Moreover, the relative abundance of *MAPK13* in the *in vitro*-derived blastocysts was greater ($P < 0.05$) than observed in the Control group. In conclusion, adding melatonin to the sperm-preparation protocol for bovine IVF improved proper fertilization and enhanced embryonic development and competence *in vitro*.

Introduction

The handling of sperm and oocyte in IVF usually is associated with oxidative stress that leads to a higher production of reactive oxygen species (ROS) during the co-incubation period [1] with potentially detrimental effects on both gametes, ultimately leading to impaired *in vitro* embryo development. The use of specific antioxidants such as melatonin to neutralize ROS could protect gametes against adverse ROS effects and may play a significant role in improving the efficiency of assisted reproductive technologies (ARTs) [2-5]. We have recently shown that melatonin enhanced bovine oocyte competence and embryo development when added during *in vitro* maturation of cumulus-oocyte-complexes from juvenile and adult donors [6].

The potent scavenging molecule melatonin (N-acetyl-5-methoxytryptamine) is critically involved in the circadian rhythm by secretion from the pineal gland, but local melatonin production has been detected in extra-pineal organs such as reproductive tissues [7-9] where it plays an essential role as an anti-oxidative molecule in intracellular regulation of ROS levels. Its accumulation in reproductive fluids such as seminal plasma [10] suggests that it could be critical for male reproductive function, including sperm motility and morphology [11], sperm viability, and fertilizing ability [12]. Nevertheless, recently it has been shown that enhanced adhesiveness of sperm to the zona pellucida and higher fertilization rates induced by melatonin, were not associated with intracellular ROS levels reduction [13].

Fertilization involves a coordinated sequence of molecular events starting with the fusion of sperm and egg, followed by fusion of both pronuclei and concomitantly the maternal and paternal chromosomes [14]. Massive epigenetic reprogramming of the paternal and maternal chromatin during fertilization and early cleavage is initiated by

maternal RNAs and proteins accumulated during oogenesis [15]. Nevertheless, the proportion of good quality embryos was significantly lower in infertile males than those with normal fertility [16], suggesting that preimplantation embryo development does not rely exclusively upon maternal regulation.

Knowledge of melatonin function on post-thaw bull semen, specifically during co-incubation of male and female gametes, and its impact on fertilization ability and *in vitro* embryo competence is scarce. Reports showed contradictory results and focused mainly on the anti-oxidative pathway [17, 18]. To date, little is known whether the putative beneficial effects of melatonin on spermatozoa also have impact on early embryo development. Enhancing gametes and embryo competence during *in vitro* embryo production has been a primary goal in ART since it allows increasing efficiency, and decreasing cost. Several studies have shown that melatonin improved embryo development *in vitro*, but insight into on the underlying mechanisms of improved embryo development is largely unknown; an anti-oxidative pathway was suggested [9, 18, 19, 20, 21, 22].

The goal of the present study was to evaluate the impact of melatonin in an established bovine IVF-protocol on basic sperm function, their ability to fertilize *in vitro*, and embryo development after IVF. To gain insight into embryo competence, we analyzed a panel of developmentally important genes involved in preimplantation embryo development, including the lineage allocation and cell polarity Cadherin 1 (*CDH1*), the caudal type homeobox 2 (*CDX2*), the regulator of cell proliferation and differentiation MAP kinase activity (*MAPK13*)], the water transport [water transport gene aquaporin 3 (*AQP3*)], the two apoptosis regulators [BCL2 Associated X, Apoptosis Regulator (*BAX*), and Heat shock protein beta-1 (*HSPB1*)], and the

pluripotency transcripts [POU domain, class 5, transcription factor 1 (*POU5F1*), and the Nanog Homeobox (*NANOG*)].

Materials and methods

Reagents, melatonin dissolving, and stock solutions

Unless otherwise stated, all reagents used in this experiment were provided by Sigma-Aldrich (Steinheim, Germany). The melatonin stock solution was prepared in TALP (Tyrode's medium base, albumin, lactate, and pyruvate) medium through serial dilution in ethanol as previously described [6]. TALP composition as previously describe in Parrish et al. [23, 24], supplemented with 6 mg/ml fatty acid free BSA (FAF-BSA, A7030-10G, Sigma-Aldrich), 0.05 mg/ml gentamicin, 0.028 mg/ml Na-pyruvate.

Three stock solutions were formed: a standard stock of plain TALP (Control), a stock for sham-treated samples containing 1% ethanol (Sham-Control), and a stock solution containing 1 nM Melatonin (MT). Aliquots of 30 µL from each stock solution were stored at -20 °C for no longer than four weeks. Working solutions contained 495 µL TALP medium plus 5 µL of a thawed stock solution to prepare medium for each of the experimental groups (Control, Sham-Control, and MT). The final concentration of melatonin and ethanol in the experiment were 0.01 nM and 0.01%, respectively. The concentration corresponds to the endogenous melatonin levels in bovine follicular fluid [25]. All working solutions were incubated for one hour at 38.5 °C and 5% CO_2 in a humidified atmosphere prior to experimentation.

Study design

This study included a set of two major experiments to evaluate the effects of an addition of melatonin to the sperm preparation protocol on sperm function, fertilization ability and embryo competence *in vitro* after use in IVF. In the first experiment, frozen-thawed semen from four Holstein-Friesian bulls with proven fertility was used. The impact of melatonin on sperm motility, acrosome integrity, and mitochondrial activity in viable sperm was determined by Computer-Assisted Semen Analysis (CASA) and fluorescence-activated cell sorting (FACS), respectively. Three experimental groups were compared by flow cytometry and CASA in conjunction with cluster analysis of single sperm data. Spermatozoa were either regularly processed (Control), processed in presence of the solvent for melatonin, i.e. ethanol, (Sham-Control) or processed in presence of melatonin (MT-Sperm). In the second experiment, semen from one of the bulls was selected to unravel putative melatonin effects on *in vitro* fertilization ability and early embryo development. The selected bull had a record of regular IVF fertility (32% blastocyst rate along 20 IVF cycles). Spermatozoa were either treated with melatonin prior to coincubation of gametes using the same experimental groups (Control, Sham-Control, MT-sperm) and one additional group where melatonin was included in the *in vitro* fertilization medium (MT-Coincubation).

Sperm preparation

Frozen-thawed semen from the same batch was used to avoid the influence of variation amongst ejaculates and cryopreserved straws [26]. Six semen straws in the first and three in the second experiment, each with a concentration of 20-25 x 10^6 spermatozoa / 0.5-ml, were thawed in a water bath at 33 °C for 1 min. Then, each straw was placed over 1.0 ml of a commercial colloidal gradient-solution for selecting and

purifying bull spermatozoa (BoviPure™, Nidacon, Mölndal, Sweeden). The Bovipure solution was prepared according to the manufacturer's instructions with slight modifications. Briefly, two concentrations of the Bovipure solution were prepared in 1.5 ml Eppendorf centrifuge tubes, one at 40% (200 µl BoviPure™ + 300 µl BoviDilute™), and the other at 80% (400 µl BoviPure™ +100 µl BoviDilute™). Prior to use, both colloidal solutions were stabilized for one hour in the incubator at 39 °C and with 5 % CO_2 in air. During thawing, the 40% solution was placed over the solution at 80%, and then semen was placed on top of this colloidal solution prior to centrifugation.

After centrifugation at room temperature at 400 *g* for nine minutes, 900 µL supernatant from each sample was removed, leaving the sperm pellet in ~100 µL colloidal solution. Then, the resuspended pellets from one bull were pooled and mixed by pipetting up and down nine times until a pure sperm-suspension was achieved that was subsequently split into aliquots of 100 µl each into three new 1.5 ml Eppendorf tubes. Each sample of purified sperm was then assigned to the Control, Sham-Control or MT-Sperm group and centrifuged for three min at 400 *g* with 500 µl of the corresponding TALP medium. After removing 300 µL supernatant, the remaining sample was homogenized and gently mixed. Then, each sperm suspension (Control, Sham-Control, MT-Sperm) was aliquoted in triplicates of 100 µL and incubated at 39 °C and 5 % CO_2, leaving the tube lid open until evaluation after 30 minutes, 120 minutes, and 180 minutes, respectively.

Sperm motility and Computer-Assisted Sperm Analysis (CASA)

In order to assess the effects of melatonin on *in vitro* sperm kinematics and motion parameters at different incubation periods, we used the IVOS® II CASA system (version 1.10, Hamilton Thorne, Beverly, USA). Prior to evaluation, the samples were

gently homogenized. Then, 10 µL of the sample was placed in a Makler chamber and analyzed. Instrument settings of the CASA system are shown in **S1 Table**.

All measurements were performed in triplicate, each analyzing five capture frames. The mean of the three replicates was used for statistical evaluation of average CASA parameters. The CASA parameters included total motility (%), progressive motility (%), straight line velocity (VSL: µm/s), curvilinear velocity (VCL: µm/s), average path velocity (VAP: µm/s), amplitude of lateral head displacement (ALH: µm), beat cross frequency (BCF: Hz), linearity (LIN: %), straightness (STR: %) and wobble (WOB: %):

Flow cytometric evaluation of spermatozoa (sperm viability, acrosome integrity, and mitochondrial activity)

Measurements of the fluorescently stained sperm were made using a Gallios™ flow cytometer (Beckman Coulter, Krefeld, Germany), equipped with three lasers for excitation at 405 nm (Violet Solid State Diode, 40 mW), 488 nm (Blue Solid State Diode, 22 mW), and 638 nm (Red Solid State, 25 mW).

In parallel to motility assessments, sperm plasma membrane integrity and acrosome integrity were assessed using combined staining with Sybr-14 / Propidium iodide (PI) from the LIVE/DEAD®Sperm Viability Kit (L-7011, Molecular Probes®, Goettingen, Germany) and fluorescently-labeled peanut agglutinin (PNA-Alexa Fluor™ 647, Invitrogen™). Additionally, the mitochondrial membrane potential (Mito Tracker™ Deep red FM, Invitrogen™) was evaluated in combination with Sybr14/PI staining to allow to discriminate viable and dead spermatozoa. Stock solutions of Sybr14 were diluted first 1:10 in DMSO kept frozen at – 20 °C and then further diluted 1:100 with Ca^{2+}-free Phosphate Buffered Saline solution (PBS), stored at 4 °C, and used

within one week. Propidium iodide stock solutions were diluted 1:10 with Ca^{2+}-free PBS (final concentration: 240 µg/ml) and stored at 4 °C. Mito Tracker™ Deep Red FM (MitoTracker Deep Red) stock solution was diluted 1:10 in DMSO kept frozen at – 20 °C and further diluted 1:100 in Ca^{2+}-free PBS as working solution (final concentration: 10 µg/ml). The working solution was stored at 4 °C and used within four weeks. A dye mix for evaluation of sperm viability and acrosome integrity (Premix 1) was prepared by mixing 5 µL Sybr14, 3 µL PI, and 5 µL of PNA-Alexa Fluor™ 647 (PNA-AF647) (final concentration: 10 µg/ml). The dye mix for evaluating mitochondrial membrane potential in viable sperm (Premix 2) was prepared by mixing 5 µL Sybr14, 3 µL PI, and 1.5 µL of MitoTracker Deep Red. After the designated incubation times, samples for determining sperm viability and acrosome integrity were prepared by adding 10 µL sperm sample to 480 µL preheated at 37 °C TRIS solution plus 9.25 µL Premix 1 (Syrb14/PI/PNA-AF647) and incubated in the dark for 15 min at 37 °C. Samples for determining sperm viability and mitochondrial membrane potential were prepared by adding 10 µL of each sperm sample to 480 µL of preheated at 37 °C TRIS solution plus 13 µL Premix 2 (MitoTracker Deep Red) and incubated in the dark for 15 min at 37 °C. Two sub-samples from treatment x times x bull combination were prepared and analyzed as technical repeats. Results of technical repeats were averaged prior to performing the statistical analysis. The flow cytometer was set at a medium flow rate for 60 seconds. Data from 10.000 spermatozoa were obtained for each sample. The machine settings and the compensation matrix are summarized in **S2 Table**. A commercial sheath fluid was used (CytoFlex Sheath Fluid, B51503, Beckman Coulter, Life Sciences, Krefeld, Germany).

The gating strategy was as follows: Forward and side scatter signals were used to discriminate single spermatozoa from debris. Subsequently, the viable sperm

population (Sybr14 positive & PI negative) with intact acrosomes (PNA-Alexa negative) was determined. In analogy, the percentage of viable spermatozoa with active mitochondria (MitoTracker Deep Red positive) was quantified. The threshold between high and low mitochondrial membrane potential was defined for samples after 15 min incubation period at 37 °C and kept constant throughout the experiment.

In vitro embryo production

In vitro maturation (IVM)

Selected cumulus-oocyte complexes (COCs) of grade 1 to 2 quality (homogeneous dark cytoplasm and at least three layers of compact cumulus cells), were collected by aspirating follicles (2-6 mm in size) from slaughterhouse-derived ovaries. COCs were incubated in groups of 25-30 in 250 µL *in vitro* maturation medium at 38.5 °C and 5% of CO_2 in a humidified atmosphere for 24 h into four-well dishes (Nunclon™ Delta Surface, Thermo Scientific, Roskilde, Denmark). The *in vitro* maturation medium contained tissue culture based-medium 199 (TCM, Sigma-Aldrich), enriched with 1 mg/ml fatty acid-free bovine serum albumin (FAF-BSA, A7030-10G, Sigma-Aldrich), and 10 IU/ml equine chorionic gonadotropin (eCG) and 5 IU/ml human chorionic gonadotropin (hCG) (Suigonan®, MSD, Intervet, Unterschleissheim, Germany).

In vitro fertilization (IVF)

After maturation, expanded COCs were divided into four groups and each placed in 250 µL fertilization medium (Fert-TALP) as previously described [23, 24], supplemented with 6 mg/ml of FAF-BSA, 10 µM hypotaurine, 1.0 IU/ml heparin, and 1.0 µM epinephrine. Three of the experimental groups were fertilized with sperm which

were prepared as described above (Control, Sham-Control, and MT-Sperm). In the fourth group, sperm from the Control group were used and melatonin was included only in the IVF medium (MT-Coincubation) to allow differentiation of effects from melatonin on the resulting embryos that originate from the co-incubation period and effects that could be ascribed to the melatonin-treated sperm. Prior to IVF, sperm samples were washed, eluted, and centrifuged for three min at 400 g with 500 µl fertilization medium (Fert-TALP), the supernatant was removed leaving the purified sperm in ~ 100 µL Fert-TALP suspension. Insemination was performed with a ratio of 100,000 sperm cells/100 µL of IVF medium (total 250,000 sperm cells per well containing 250 µL of IVF medium). Semen quality of samples from the selected bull was as follows: Total motility: 71.0±2.0%, Progressive motility: 69.2±1.9%, viable sperm with high mitochondrial membrane potential: 77.7±1.9%, viable sperm with intact acrosome: 42.5±2.1%. All values are from the Control group after 15 minutes incubation. A comparison of all bulls is shown in **S3 Table**.

In vitro culture (IVC)

After IVF (18-20 h), cumulus cells from the presumptive zygotes were removed by vortex the oocytes in tissue culture medium (TCM) 199 medium with HEPES modification (TCM medium 199, M252, Sigma-Aldrich) at 3.000 rpm for five minutes and placed into four-well dishes containing 500 µL *in vitro* culture (IVC) medium based on synthetic oviductal fluid (SOF) [27, 28], enriched with 4 mg/ml of FAF-BSA-covered with 600 µL mineral oil (GYNEMED, Lensahn, Germany), incubated at 38.5 °C, 5% of CO_2 and 5% of O_2 in a humidified atmosphere, until blastocyst formation (day 7.5). Unlike IVC, both IVM and IVF system were oil-free.

IVF outcomes were evaluated by determining rates of cleavage, blastocysts, and advanced blastocysts. Cleavage rate was determined 72 h post-insemination (hpi), and blastocysts rate at 180 hpi (day 7.5) as the percentage of cleaved zygotes. Early and non-expanded blastocysts (mid blastocysts) were graded as blastocysts; while expanded, hatching, and hatched blastocysts as advanced blastocysts. Six replicates of *in vitro* embryo production were performed.

Determination of fertilization

The fertilization rate was determined by pronuclear (PN) assessment using lacmoid staining according to an established protocol in our lab. A lacmoid stock solution was prepared by dissolving 1.0 g lacmoid powder (CarlROTH®, Karlsruhe, Germany) in 45 ml slightly heated acetic acid. Then, a working solution was prepared by adding 5.0 ml from the stock solution to 5.5 ml of H_2O (Milli-Q®).

After 18-20 h IVF, cumulus cells from the presumptive zygotes were removed by vortexing the oocytes in TCM medium at 3.000 rpm for five minutes, followed by washing in phosphate-buffered saline (PBS, Sigma-Aldrich) supplemented with 1% polyvinyl alcohol (PVA, Sigma-Aldrich) (PBS-PVA). Next, 5 to 6 zygotes were mounted into small drops of PBS-PVA (approx. 0.3 µL) on a glass slide between two parallel lines of paraffine and covered with a coverslip. This procedure was performed under a stereomicroscope at room temperature. Afterward, slides were put into fixative solution (one-part of acetic acid: three parts of ethanol 100%) for 24 h until evaluation. Finally, the slides were removed from the fixative solution and the lacmoid working solution gently infused between slide and coverslip with an insulin syringe. The pronuclei were visually inspected by using phase-contrast microscopy (Olympus BH2, Tokyo, Japan) switching different-fold magnifications amongst the objectives.

Regular fertilization was defined when two pronuclei (2 PN) were visible. Fertilization failure was diagnosed when either one or no pronuclei were identified. Polyspermy was assumed when three or more pronuclei were identified (**Fig 1**). Zygotes from three different IVF cycles were evaluated.

Gene expression analysis in *in vitro*-derived embryos

Quantification of the relative expression of the developmental genes Cadherin 1 (*CDH1*), caudal type homeobox 2 (*CDX2*), the regulator of MAP kinase activity (*MAPK13*), the water transport gene aquaporin 3 (*AQP3*), the apoptotic regulators BCL2 Associated X, Apoptosis Regulator (*BAX*), and Heat shock protein beta-1 (*HSPB1*), and the pluripotent POU domain, class 5, transcription factor 1 (*POU5F1*), and the Nanog Homeobox (*NANOG*) was performed using the primers listed in **Table 1**.

RNA Preparation

Poly(A)+ RNA was isolated using the Dynabeads® mRNA Direct Kit (Life Technologies) according to the manufacturer's instruction with some modifications. Briefly, pools of three expanded blastocysts each from the different experimental groups (Control: *n*: 6, Sham-Control: *n*: 9, MT-Sperm: *n*: 7, MT-Co-incubation: *n*: 7) were thawed in 40 µl of lysis-binding buffer (100 mM Tris-HCL, at pH 8.0, 500 mM LiCl, ten (10) mM EDTA, 1% LiDS, 5.0 mM DTT). Then 3.0 pg rabbit globin mRNA was added as internal standard and incubated at room temperature for 10 minutes. Prewashed Dynabeads® Oligo d(T)25 (5 µl) were pipetted to the lysate and incubated for 15 minutes at 25 °C on a shaker for binding poly(A)+ RNA to the beads. The beads were separated by using a Dynal MPC-E-1 magnetic separator, washed once with washing buffer A (10 mM Tris-HCL. pH 8.0, 0.15 mM LiCl, 1.0 mM EDTA, 0.1%

LiDS) and two times with washing buffer B (10 mM Tris-HCL. pH 8.0, 0.15 mM LiCl, 1.0 mM EDTA). The poly(A)+ RNA was eluted from the beads by incubation in 10 µl sterile water at 68 °C for two minutes, and the mRNA was used immediately as input for the reverse transcription reaction.

Reverse transcription (RT)

Reverse transcription (RT) was conducted in a 20 µl volume consisting of 4.0 µl of 5x RT buffer (Life Technologies), 1.0 mM dNTP (Life Technologies), 20 Units RNAsin® (Life Technologies), 100 Units M-MLV reverse transcriptase (Life Technologies, Cat.no. 28025-013) and 2.5 µM random hexamer primer (Life Technologies). Afterward, samples were incubated at 25°C for ten minutes for primer annealing and then incubated at 42°C for one hour. Finally, the samples were heated to 90°C for 5 minutes. The cDNA was diluted to a concentration of 0.1 embryo equivalent/µl. Two µl were used for Real-Time PCR amplification.

Real-time qPCR

Real-time qPCR was carried out in 96-Well Optical Reaction Plates (Life Technologies). The PCR mix in each well included 10 µl of 2x Power SYBR_Green PCR Master Mix (Life Technologies, Cat.no. A25742), and 0.1 µM of each of the forward and reverse primers and 2.0 µl cDNA in a final reaction volume of 20 µl. The PCR reaction was carried out in an ABI 7500 Fast Real-Time System (Applied Biosystems) using the following program: denaturation and activation of the Taq Polymerase during 10 min at 95 °C followed by 40 cycles at 95 °C during 15 seconds and 60 °C for one minute, followed by slow heating for displaying a dissociation curve of the product. Data generated by the Sequence Detection Software 1.4 were transferred

to Microsoft Excel for analysis. Differential mRNA expression of each target gene was calculated by the Relative Standard Curve Method. A cDNA dilution from pooled blastocysts mRNA was included on every plate to yield standard curves for each gene. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and rabbit globin were used as the internal controls. Standard curves were used to calculate the relative concentration of each target gene to be normalized to the geometric mean from Globin/Gapdh expression results for each sample.

Statistical analysis

General statistics

Data were statistically analyzed using the JMP software (Version 15; SAS Institute, Inc., Cary, NC, 2015) and the Statistical Analysis package (SAS)® (Version 9.4, Cary, NC, USA). Significant differences were defined at $P < 0.05$ except for gene expression analysis where a potential statistical trend was also considered ($P \leq 0.10$). All data are expressed as LS mean ± SEM (standard error of the mean) unless otherwise stated.

Sperm motility assessment

Prior to analysis, a test of normality was assessed through Shapiro Wilk W test and unequal variance through Levine test. To evaluate the effects of treatment and incubation time (30-min, 120-min, and 180-min) on sperm motility and sperm kinetics a linear mixed model fit by Restricted Maximum Likelihood (REML) was applied (JMP). To consider repeated measures, bull was considered as random effect, while treatment and incubation time and their interaction were treated as fixed effects. Tukey multiple pairwise comparisons were used to compare differences between experimental groups.

In addition, a sperm subpopulations analysis (cluster analysis) was performed to identify putative changes in motility patterns due to treatment or incubation time. Cluster analysis considered the data from eight motility descriptors (VSL, VCL, VAP, ALH, BCF, LIN, STR, and WOB) for every single motile sperm (n = 153.016). Data from all incubation times and media were combined in one data set. After checking for normality, parameters were correlated (PROC UNIVARIATE and PROC CORR, SAS®). Only variables correlating <0.9 (VSL, VCL, VAP, ALH, BCF, LIN, STR, and WOB) were selected for the clustering procedure. Chosen variables were standardized to a mean of 0 and a standard deviation of 1 to avoid bias in the clustering procedure. Then, a hierarchical clustering method was conducted using squared Euclidean distance as distance measurement and the 'centroid' algorithm for cluster fusion (PROC CLUSTER, SAS®). The choice of a suitable solution was guided by the cubic clustering criterion (CCC), pseudo-F statistics, and pseudo-t^2 values. A Pearson Chi-square test was used to compare distribution of sperm to the different clusters between time points and treatments (PROC FREQ, SAS®). Cramer's V (ranging from 0 to 1) was used, to evaluate effects from factors treatment or time. Results were interpreted in analogy to the proposed guidelines (Cohen, 1988): $V < 0.10$ = no effect, $0.10 < V \leq 0.30$ = slight effect, $0.30 < V \leq 0.50$ = moderate effect, $V > 0.50$ = large effect.

Fertilization test and IVF outcomes

To analyze the effect of treatment on the distribution of regularly fertilized and polyspermic oocyte penetration a Generalized Linear Model (GLM) from JMP (SAS®) with Chi-Square statistic test was used considering a Poisson distribution model of fertilization and sperm-egg interaction *in vitro* as previously suggested [29]. The other categorical data from IVF outcomes and embryo developmental rates (e.g., cleavage,

blastocysts, and advanced blastocysts) were compared using the logistic procedure (PROC LOGISTIC, SAS®). Analysis of maximum likelihood estimates was performed to determine the odds ratio estimates, the confidence interval limits for relative risks of embryo developmental rates through the logistic procedure from SAS®.

Gene expression in *in vitro*-derived expanded blastocysts

The influence of treatment on gene expression was analyzed using a Generalized Linear Model (GLM) from JMP (SAS®). Test of normality was assessed through Shapiro Wilk W test and unequal variance through Levine test. All data are expressed as the LS mean values for each set of data ± SEM.

Results

Sperm motility

The effects of treatment on sperm motility and CASA parameters are presented in **Table 2**. The overall means for the three different experimental groups (Control, Sham-Control, MT-Sperm) show that significant effects of melatonin on overall motility or sperm kinematics were not evident. The LS means for MT-Sperm were higher for total motility, progressive motility, VCL, and VSL when compared to the Control group ($P < 0.05$), but did not differ from Sham-Control samples ($P > 0.05$).

Sperm motility parameters were markedly affected by the incubation period (**Table 3**). The impact of the incubation time resulted in a significantly decreased ($P < 0.05$) percentage of total motility and progressive motility spermatozoa after 120 min and 180 min of incubation. Similarly, a significant decrease ($P < 0.05$) in the LS means

of BCF, VCL was observed, while the average levels for VSL and LIN were significantly increased ($P < 0.05$).

The interaction between treatment and incubation time was not significant ($P > 0.05$) at any of the traits evaluated (**S4 Table**).

Cluster analysis of the detected sperm subpopulations (SPs)

Clusters analysis results are summarized in **Fig 2**. A solution with five main sperm subpopulations (SPs; SP1 to SP5) was chosen which explained 82.9 % of the variance in the dataset. A cluster was considered as main cluster, if at any combination of treatment by incubation time ≥ 5% of the spermatozoa were assigned to it. A summary of the motility descriptors for the individual cluster is provided in **Fig 2A**. No general treatment effect ($P > 0.05$; Cramer's V: 0.02) was observed on the distributions of sperm subpopulations amongst experimental groups (**Fig 2B**). A significant overall effect of incubation time was detectable, although the effect of incubation time was estimated to be relatively small ($P < 0.05$; Cramer's V: 0.19; **Fig 2C**). Nonetheless, the change between 30 min and 180 min incubation time was characterized by a clear decrease in spermatozoa with fast VCL, wide ALH and low LIN (SP3: 23.1% to 9.2% and SP5: 12.7 % to 6.2 %) and a concomitant increase in spermatozoa with moderate kinematic parameters (SP2: 32.2 % to 60.1 %). A stratified view of the data confirmed that the melatonin treatment did not have an effect on sperm distribution to the different subpopulations at any incubation time (**Fig 2D**).

Sperm viability, acrosome integrity, and mitochondrial activity

Assessment of sperm quality and function by flow cytometry did not reveal significant ($P > 0.05$) effects of treatment or incubation time on the evaluated

parameters (**Table 4**) and the interaction between treatment and incubation time (**S5 Table**).

Determination of fertilization

Chi-Square testing revealed a significant increase ($P < 0.01$) of the percentage of regular, i.e. monospermic fertilization in COCs exposed to sperm pre-treated with melatonin during the sperm preparation protocol (MT-Sperm: 82.8%) compared to that found in the Control (66.6%) and Sham-Control (50.0%) groups **(Table 5)**. Similarly, the group MT-Co-incubation had a greater proportion of regularly fertilized COCs than Sham-Control (71.9% vs. 50%, respectively), but not ($P > 0.05$) when compared with the Control or MT-Sperm (71.9% vs. 66.6% and 82.8%, respectively). Regular fertilization was also greater for the Control (66.6%) ($P < 0.05$) compared to Sham-Control (50%). On the other hand, the percentage of zygotes with polyspermy was greater ($P < 0.01$) in the control groups (Control: 16.6%, Sham Control: 23.3%) compared to that found in the melatonin groups (MT-Sperm: 6.9%, MT-Co-incubation: 9.4%).

In vitro embryo production

Logistic regression analysis revealed similar cleavage rates ($P > 0.05$) amongst the experimental groups (Control: 73.7%, Sham-Control: 80.3%, MT-Sperm: 79.3%, MT-Co-incubation: 79.6% (**Table 6**). However, the blastocyst rate from the MT-Sperm group was greater ($P < 0.05$) than that from the Control group (54.8% vs. 39.1%). Logistic regression analysis showed that the odds ratio (OR) for blastocyst formation of COCs fertilized with sperm pre-treated with melatonin was 1.88 times more likely to become blastocysts than for COCs inseminated in the Control groups (95% confidence

intervals: 1.115 - 3.180). Similarly, the percentage of advanced blastocysts (expanded, hatching, and hatched blastocysts) was greater ($P < 0.05$) in the MT-Sperm (33.0%) group compared to the Control group (20.9%). Moreover, the OR showed that addition of melatonin to the sperm preparation protocol made it 1.87 times more likely to produce advanced stages of the embryos than the Control group (95% confidence intervals: 1.033 - 3.390). Statistical analysis did not reveal differences in the blastocyst rate and the proportion of advanced embryo development in both, Control and Sham-Control group; the same was true for the MT-Sperm and MT-Co-incubation groups, respectively.

Gene expression analysis and embryo competence from the *in vitro*-derived embryos

Results from the gene expression analysis are summarized in **Fig 3**. The relative abundance of the regulator of MAP kinase activity (*MAPK13*) was significantly greater ($P < 0.05$) than observed in the Control group, but only marginally different from that found in the Sham-Control ($P = 0.06$) and MT-Co-incubation ($P = 0.07$) groups. The expression of the other genes related to developmental pathways [Cadherin 1 (*CDH1*), caudal type homeobox 2 (*CDX2*)], and water transport [aquaporin 3 (*AQP3*)] did not differ ($P > 0.05$) between treatments.

Transcript levels of the pro-apoptotic BCL2 Associated X, Apoptosis Regulator (*BAX*) gene, were lower in the MT-Co-incubation group compared to the Control group ($P < 0.05$), albeit marginal statistical significance in the model ($P =0.1$) was observed. The mRNA abundance of the gene Heat shock protein beta-1 (*HSPB1*) did not differ amongst experimental groups ($P > 0.05$).

The relative abundance of mRNA for the pluripotency-related genes POU domain, class 5, transcription factor 1 (*POU5F1*), and Nanog homeobox (*NANOG*) was not significantly different amongst embryos derived from sperm derived from the different treatment groups ($P > 0.05$).

Discussion

Here, we demonstrate that melatonin treatment of spermatozoa prior to or at the time of gamete coincubation improved monospermic fertilization rate and was beneficial for early embryo development up to the blastocyst stage after IVF. We also observed that melatonin treatment resulted in an increased proportion of advanced blastocysts (expanded, hatching, and hatched blastocysts), indicating that embryos derived from fertilization with melatonin-treated sperm may have an improved developmental competence.

Increased monospermic fertilization, as observed in the present study, unveils melatonin's potential to improve current *in vitro* fertilization systems. For example, a high incidence of polyspermy is still an unresolved problem for *in vitro* embryo production in the pig [30], while in bovine IVF, it is considered as less common problem with unclear etiology [31]. Although polyspermy has been attributed to mechanisms regulated mainly by the oocyte [32], strategies implemented at the sperm level have successfully reduced its occurrence. For instance, high pre-freezing sperm dilution in porcine IVP increased monospermy, cleavage rate, and blastocyst formation [30]. Other researchers [33] found a significant reduction of polyspermic oocytes when boar spermatozoa were encapsulated and preserved in barium alginate membranes. However, it has to be taken into account, that despite numerous efforts porcine IVF is still prone to polyspermic fertilization [34, 35. 36]. Thus, our results could serve as a

practical strategy to increase monospermic fertilization in bovine and porcine IVF systems.

In a recent study using boar sperm under capacitating conditions, melatonin reduced the polyspermy rate, increased the proportion of spermatozoa binding to the zona pellucida, and regulated *in vitro* sperm capacitation (IVC) and subsequent progesterone-induced acrosome exocytosis (IVAE) via changes in the number of tail disulfide bridges [13]. The authors also found that melatonin dramatically decreased motility of capacitated sperm, with a maximal effect at 5 μm [13]. It is not clear how melatonin decreased the occurrence of polyspermy in our study. However, it is necessary to consider that regardless of *in vivo* or *in vitro* conditions, a high concentration of capacitated spermatozoa at the fertilization site is considered to be involved in an increased proportion of polyspermy [37]. Under capacitating conditions bull spermatozoa exposed to melatonin had reduced progressive motility and curvilinear velocity (VCL), inhibiting sperm capacitation [38]. Modulation of sperm capacitation by melatonin has been linked to reduced cAMP levels [39], and presumably could at least in part explain the reduced percentage of polyspermic zygotes after melatonin treatment found in our study.

Other studies [40] observed that even though melatonin failed to affect sperm kinematic parameters and viability in ram spermatozoa, exposure to 100 pm melatonin increased cleavage rate following IVF compared to the control sample. The authors also found a dose-dependent effect on sperm capacitation and subsequent fertilization rate. Decreased sperm capacitation and phosphatidylserine translocation were observed at 1 μm, whereas increasing short-term capacitation at 100 pm affected fertilization (100 pm: 99.2% vs. 1 μm: 92.6%) and cleavage rates (1 μm: 79.6% vs. 100 pm: 88.6%). By contrast, addition of 1 mmol melatonin to IVF media was deleterious for embryo

development (only 2% blastocysts), while lower melatonin concentrations (0.01 mM and 0.1 mM) had no adverse effects but did not significantly improve *in vitro* embryo production compared to the control group (control: 23.0%; melatonin 0.01 mM: 5.7%; melatonin 0.1 mM: 9.6%) [17]. Deleterious effects could be related to the higher melatonin concentration (1-100K times higher) in the previous study [17] compared to the current experiment (0.01 nM). The endogenous melatonin levels in bovine follicular fluid were determined to be 10^{-11} M (0.01nM) [25], similar to the concentration we used in the present study.

Previous studies had shown beneficial effects of melatonin on sperm motility under different conditions in various species. For instance, short-term exposure *in vitro* to 1.0 mM of melatonin had a favorable impact on human sperm function by enhancing various sperm motility parameters [11]. Likewise, melatonin supplementation in bull semen extender increased kinetic parameters such as total motility, progressive motility, linearity, sperm track straightness, lateral head displacement, and also sperm viability and functionality after freezing-thawing [41]. Albeit we observed that melatonin improved some sperm kinematics traits (incl. total motility, progressive motility, VCL, and VSL) compared to the control, no significant differences were observed against the sham control (Ethanol).

Other investigators found that melatonin had dose-dependent adverse effects on sperm motility and forward progression in rats, which was partially attributed to ethanol as a solvent solution (0.5% final concentration) [42]. In our experiment, the final ethanol concentration (0.01%) did not negatively affect total sperm motility and progressive motility. Additionally, a high melatonin concentration (1000 nmol) was associated with a lower percentage of sperm with intact acrosomes, fewer viable spermatozoa with ROS, higher DNA fragmentation, and DNA oxidation, whereas 10

nmol had higher ROS levels than controls [17]. Albeit the underlying mechanisms by which higher the melatonin concentration increased ROS in that experiment remained unclear; presumptive pathways were either via (1) calmodulin-mediated and/or (2) calcium-independent phospholipase A2 (iPLA2-mediated) which may be involved in the stimulation of ROS's production by melatonin [43].

In the present experiment, sperm plasma membrane, acrosome integrity, and mitochondria membrane potential were not affected by melatonin treatment at the employed concentration (10^{-11} M) which is in contrast to the work performed previously [17]. On the contrary, it has been found that melatonin supplementation (10^{-5} and 10^{-3} M) protects sperm from ROS-associated damage and increased semen quality after freezing-thawing [44]. Similarly, other investigators observed improved cell viability, plasma membrane integrity, mitochondrial activity, acrosome integrity, reduced intracellular ROS levels, and subsequent embryo development of embryos originated from melatonin-treated spermatozoa [18].

Only one study had demonstrated beneficial effects of melatonin on post-thaw bovine sperm functionality and *in vitro* embryo development [18]. Although the *in vitro* sperm kinematics parameters were not improved in the referred experiment, sperm treatment with melatonin (10^{-3} M) resulted in an enhanced blastocyst rate [18]. Results presented in the current study indicate that oocytes fertilized with melatonin-treated sperm have an increased likelihood to become blastocysts compared to the controls. Moreover, a higher proportion of embryos reaching advanced embryo development stages (expanded, hatching, and hatched blastocysts), was also increased after melatonin treatment, indicating improved embryo competence and better embryo quality.

In vitro-derived embryos usually are less competent than their *in vivo*-derived counterparts, reflected in lower pregnancy rates, with ~60% of these pregnancies failing during the first six weeks of gestation [45] clearly indicating reduced embryo competence of IVP derived embryos. Embryonic cell counting as an indirect indicator of embryo competence revealed that a reduced number of trophectoderm (TE) cells may be associated with embryo losses, while decreased number of inner cell mass (ICM) cells is thought to increase the risk of fetal mortality or impaired development [46]. Recently, we found that adult and prepubertal oocytes, during *in vitro* maturation medium supplemented with melatonin possessed an increased number of ICM, and total embryonic cells, and showed a higher proportion of advanced blastocysts [6]. Some features involved in the lower competence of *in vitro*-derived embryos included a reduced number of embryonic cells, less elongated conceptus, smaller embryonic disk, compromised yolk sac development, marginal development of binucleate cells and cotyledon, and reduced placenta vascularization [45].

The gene expression analysis in our study revealed that expanded blastocysts derived from melatonin treated-sperm had increased *MAPK13* gene expression when compared to the controls. Early bovine embryos critically depend on both MAP kinase signaling and the extracellular signal-regulated kinase (ERK) pathway to complete development to the blastocyst stage [47]. Recently, it was found that MAP kinase signaling was crucial for ICM differentiation via restricting epiblast cell numbers [48]. Increased epiblast precursors and decreased hypoblast precursors were observed when MAP kinase signaling was inhibited in bovine embryos [49]. Quantification of the number of cells expressing cell lineage segregation transcripts revealed an increase in the proportion of *NANOG*-positive cells while decreasing *GATA*-positive ones in the ICM of embryos that were cultured in the presence of a MAP kinase inhibitor [49].

NANOG-positive cells are destined to become the pluripotent epiblast, whereas *GATA*-positive cells likely become the differentiated hypoblast, also known as the primitive endoderm [50]. We did not measure expression of *GATA* in the current study, nonetheless, increased *MAPK13* expression did not affect the transcription level of *NANOG*, *POU5F1* (required for *NANOG* expression in the bovine blastocyst [51], and the TE cell segregation transcript *CDX2* [52].

The MAP kinase pathway may play an essential role in keeping the balance in embryonic cell lineage segregation, which is critical to maintain a physiological number of hypoblast cells. The yolk sac, which develops from the hypoblast, is indispensable in early pregnancy in all mammals playing an essential role in histotroph digestion [revised in 45]. ERK/MAPK signaling is required for proper growth, differentiation, and morphogenesis of the placenta in the mouse [53]. In the bovine, the growth of epidermal primary trophoblast cells was accelerated in medium supplemented with epidermal growth factor (EGF) via activation of RAS and phosphorylation of MAPK [54].

Recently, it was shown that a lower level of the microRNA miR-216b in bovine spermatozoa and zygotes was associated with a higher level of K-RAS (a gene of the RAS/MAPK pathway) in two-cell embryos, which increased first cleavage rate and blastocyst cell numbers from bulls with high fertility, while the opposite was for low fertility bulls [55]. Similarly, transcriptomic profiling of buffalo spermatozoa revealed downregulation of 28 genes associated with MAPK signaling pathway bulls with low fertility compared to those with high fertility [56]. Thus the enhanced fertilization ability and embryo development observed in our study could hypothetically be linked to this pathway. The underlying molecular mechanisms of melatonin-treated sperm modulated MAPK signaling in the *in vitro*-derived blastocysts warrant further investigation. Better sperm protection from apoptosis via the melatonin receptor and

extracellular signal-regulated kinase (ERK) in human ejaculates has been reported [57]. MAPK is involved in several male reproductive functions, incl- sperm motility, hyperactivation, capacitation, and acrosome reaction prior to fertilization in the female reproductive tract [58].

Finally, downregulation of the expression of the pro-apoptotic regulator BAX gene observed in embryos derived from melatonin (MT-Coincubation) in the current experiment agrees with a previous report in which melatonin improved embryo quality due to a lower number of apoptotic cells and an enhanced anti-apoptotic and antioxidant gene expression profile in the embryos [18]. The anti-apoptotic and anti-oxidative effects of melatonin may have reduced the oxidative stress on spermatozoa and enhanced bovine embryo quality [18]. The other genes analyzed in the present study were not significantly affected by melatonin treatment, including the lineage allocation and cell polarity Cadherin 1 (*CDH1*) and the water transport gene aquaporin 3 (*AQP3*).

In conclusion, adding melatonin to the sperm-preparation protocol for IVF enhanced sperm fertilization ability and embryo development *in vitro.* The current work suggests that the spermatozoa may play a more active role in regulating early embryo development fate than initially thought. Hence, further studies to explore this assumption are warranted.

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References

1. Agarwal A, Durairajanayagam D, du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol. 2014; 24;12:112. doi: 10.1186/1477-7827-12-112. PMID: 25421286; PMCID: PMC4258799.
2. Tamura H, Jozaki M, Tanabe M, Shirafuta Y, Mihara Y, Shinagawa M, et al. Importance of Melatonin in Assisted Reproductive Technology and Ovarian Aging. Int J Mol Sci. 2020; 21(3):1135. doi: 10.3390/ijms21031135. PMID: 32046301; PMCID: PMC7036809.
3. Al-Gubory KH, Bolifraud P, Germain G, Nicole A, Ceballos-Picot I. Antioxidant enzymatic defence systems in sheep corpus luteum throughout pregnancy. Reproduction. 2004 Dec;128(6):767-74. doi: 10.1530/rep.1.00389. Erratum in: Reproduction. 2005; 129(1):127. Ceballos-Bicot, Irène [corrected to Ceballos-Picot, Irène]. PMID: 15579594.
4. Al-Gubory KH, Garrel C, Faure P, Sugino N. Roles of antioxidant enzymes in corpus luteum rescue from reactive oxygen species-induced oxidative stress. Reprod Biomed Online. 2012; 25(6):551-60. doi: 10.1016/j.rbmo.2012.08.004. Epub 2012 Sep 12. PMID: 23063822.
5. Tamura H, Takasaki A, Taketani T, Tanabe M, Lee L, Tamura I, et al. Melatonin and female reproduction. J Obstet Gynaecol Res. 2014; 40(1):1-11. doi: 10.1111/jog.12177. Epub 2013 Oct 7. PMID: 24118696.
6. Gutiérrez-Añez JC, Lucas-Hahn A, Hadeler KG, Aldag P, Niemann H. Melatonin enhances in vitro developmental competence of cumulus-oocyte complexes collected by ovum pick-up in prepubertal and adult dairy cattle. Theriogenology.

2021; 161:285-93. doi: 10.1016/j.theriogenology.2020.12.011. Epub 2020 Dec 11. PMID: 33360610.

7. Rocha CS, Rato L, Martins AD, Alves MG, Oliveira PF. Melatonin and male reproductive health: relevance of darkness and antioxidant properties. Curr Mol Med. 2015; 15(4):299-11. doi: 10.2174/1566524015666150505155530. PMID: 25941822.
8. Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC, et al.. Extrapineal melatonin: sources, regulation, and potential functions. Cell Mol Life Sci. 2014; 71(16):2997-25. doi: 10.1007/s00018-014-1579-2. Epub 2014 Feb 20. PMID: 24554058.
9. Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, et al. Melatonin as a free radical scavenger in the ovarian follicle. Endocr J. 2013; 60(1):1-13. doi: 10.1507/endocrj.ej12-0263. Epub 2012 Dec 22. PMID: 23171705.
10. Kratz EM, Piwowar A. Melatonin, advanced oxidation protein products and total antioxidant capacity as seminal parameters of prooxidant-antioxidant balance and their connection with expression of metalloproteinases in context of male fertility. J Physiol Pharmacol. 2017; 68(5):659-68.
11. Ortiz A, Espino J, Bejarano I, Lozano GM, Monllor F, García JF, et al. High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. J Pineal Res. 2011; 50(2):132-9. doi: 10.1111/j.1600-079X.2010.00822.x. Epub 2010 Oct 21. PMID: 20964711.
12. Zhang XY, Xiong YM, Tan YJ, Wang L, Li R, Zhang Y, et al. Melatonin rescues impaired penetration ability of human spermatozoa induced by mitochondrial

dysfunction. Reproduction. 2019; 158(5):465-75. doi: 10.1530/REP-19-0231. PMID: 31505459.

13. Rocco M, Betarelli R, Placci A, Fernández-Novell JM, Spinaci M, Casao A, et al. Melatonin affects the motility and adhesiveness of in vitro capacitated boar spermatozoa via a mechanism that does not depend on intracellular ROS levels. Andrology. 2018; 6(5):720-36. doi: 10.1111/andr.12504. Epub 2018 Jun 1. PMID: 29858528.
14. Georgadaki K, Khoury N, Spandidos DA, Zoumpourlis V. The molecular basis of fertilization (Review). Int J Mol Med. 2016; 38(4):979-86. doi: 10.3892/ijmm.2016.2723. Epub 2016 Aug 31. PMID: 27599669; PMCID: PMC5029953.
15. Schultz RM. Regulation of zygotic gene activation in the mouse. Bioessays. 1993; 15(8):531-538. doi: 10.1002/bies.950150806. PMID: 8135766.
16. Gao Y, Cheng H, Gen Y, Mao G, Liang Y, Li H. Effect of semen quality on the embryo development. J Huazhong Univ Sci Technolog Med Sci. 2006; 26(1):127-9. doi: 10.1007/BF02828058. PMID: 16711026.
17. Cheuquemán C, Arias ME, Risopatrón J, Felmer R, Álvarez J, Mogas T, et al. Supplementation of IVF medium with melatonin: effect on sperm functionality and in vitro produced bovine embryos. Andrologia. 2015; 47(6):604-15. doi: 10.1111/and.12308. Epub 2014 Jul 25. PMID: 25059349.
18. Pang YW, Sun YQ, Jiang XL, Huang ZQ, Zhao SJ, Du WH, et al. Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. Mol Reprod Dev. 2016; 83(11):993-02. doi: 10.1002/mrd.22742. Epub 2016 Oct 10. PMID: 27653174.

19. Tanabe M, Tamura H, Taketani T, Okada M, Lee L, Tamura I, et al. Melatonin protects the integrity of granulosa cells by reducing oxidative stress in nuclei, mitochondria, and plasma membranes in mice. J Reprod Dev. 2015; 61(1):35-41. doi: 10.1262/jrd.2014-105. Epub 2014 Nov 1. PMID: 25366368; PMCID: PMC4354229.

20. Soto-Heras S, Roura M, Catalá MG, Menéndez-Blanco I, Izquierdo D, Fouladi-Nashta AA, et al. Beneficial effects of melatonin on in vitro embryo production from juvenile goat oocytes. Reprod Fertil Dev. 2018; 30(2):253-61. doi: 10.1071/RD17170. PMID: 28625274.

21. Soto-Heras S, Catalá MG, Roura M, Menéndez-Blanco I, Piras AR, Izquierdo D, et al. Effects of melatonin on oocyte developmental competence and the role of melatonin receptor 1 in juvenile goats. Reprod Domest Anim. 2019; 54(2):381-90. doi: 10.1111/rda.13378. Epub 2018 Dec 4. PMID: 30444551.

22. Li CY, Hao HS, Zhao YH, Zhang PP, Wang HY, Pang YW, et al. Melatonin Improves the Fertilization Capacity of Sex-Sorted Bull Sperm by Inhibiting Apoptosis and Increasing Fertilization Capacitation via MT1. Int J Mol Sci. 2019; 20(16):3921. doi: 10.3390/ijms20163921. PMID: 31409031; PMCID: PMC6720564.

23. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology. 1986;25(4):591-600. doi: 10.1016/0093-691x(86)90143-3. PMID: 16726150.

24. Parrish JJ. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. Theriogenology. 2014; 81(1):67-73. doi: 10.1016/j.theriogenology.2013.08.005. PMID: 24274411; PMCID: PMC3886814.

25. Tian X, Wang F, He C, Zhang L, Tan D, Reiter RJ, et al. Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. J Pineal Res. 2014; 57(3):239-47. doi: 10.1111/jpi.12163. Epub 2014 Aug 25. PMID: 25070516.
26. Valverde A, Arenán H, Sancho M, Contell J, Yániz J, Fernández A, et al. Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws. Asian J Androl. 2016; 18(6):851-57. doi: 10.4103/1008-682X.187579. PMID: 27678464; PMCID: PMC5109875.
27. Tervit HR, Whittingham DG, Rowson LE. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil. 1972; 30(3):493-97. doi: 10.1530/jrf.0.0300493. PMID: 4672493.
28. Gandhi AP, Lane M, Gardner DK, Krisher RL. A single medium supports development of bovine embryos throughout maturation, fertilization and culture. Hum Reprod. 2000; 15(2):395-401. doi: 10.1093/humrep/15.2.395. PMID: 10655312.
29. Aitken RJ, Elton RA. Significance of Poisson distribution theory in analysing the interaction between human spermatozoa and zona-free hamster oocytes. J Reprod Fertil. 1984; 72(2):311-21. doi: 10.1530/jrf.0.0720311. PMID: 6512759.
30. Martinez CA, Cambra JM, Maside C, Cuello C, Roca J, Martinez EA, et al. High pre-freezing sperm dilution improves monospermy without affecting the penetration rate in porcine IVF. Theriogenology. 2019; 131:162-68. doi: 10.1016/j.theriogenology.2019.04.001. Epub 2019 Apr 3. PMID: 30974393.
31. Papi M, Brunelli R, Familiari G, Frassanito MC, Lamberti L, Maulucci G, et al. Whole-depth change in bovine zona pellucida biomechanics after fertilization: how relevant in hindering polyspermy? PLoS One. 2012; 7(9):e45696. doi:

10.1371/journal.pone.0045696. Epub 2012 Sep 26. PMID: 23049839; PMCID: PMC3458926.

32. Coy P, Avilés M. What controls polyspermy in mammals, the oviduct or the oocyte? Biol Rev Camb Philos Soc. 2010; 85(3):593-605. doi: 10.1111/j.1469-185X.2009.00117.x. Epub 2009 Dec 23. PMID: 20039874.
33. Faustini M, Bucco M, Galeati G, Spinaci M, Villani S, Chlapanidas T, et al. Boar sperm encapsulation reduces in vitro polyspermy. Reprod Domest Anim. 2010; 45(2):359-62. doi: 10.1111/j.1439-0531.2008.01213.x. Epub 2008 Dec 19. PMID: 19144017.
34. Funahashi H. Polyspermic penetration in porcine IVM-IVF systems. Reprod Fertil Dev. 2003; 15(3):167-77. doi: 10.1071/rd02076. PMID: 12921703.
35. Gil MA, Almiñana C, Roca J, Vázquez JM, Martínez EA. Boar semen variability and its effects on IVF efficiency. Theriogenology. 2008; 70(8):1260-8. doi: 10.1016/j.theriogenology.2008.06.004. Epub 2008 Aug 3. PMID: 18676010.
36. Romar R, Funahashi H, Coy P. In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years. Theriogenology. 2016; 85(1):125-34. doi: 10.1016/j.theriogenology.2015.07.017. Epub 2015 Jul 23. PMID: 26271164.
37. Wang WH, Day BN, Wu GM. How does polyspermy happen in mammalian oocytes? Microsc Res Tech. 2003 Jul 1;61(4):335-41. doi: 10.1002/jemt.10346. PMID: 12811738.
38. Fernández-Alegre E, Álvarez-Fernández I, Domínguez JC, Casao A, Martínez-Pastor F. Melatonin Non-Linearly Modulates Bull Spermatozoa Motility and Physiology in Capacitating and Non-Capacitating Conditions. Int J Mol Sci. 2020; 21(8):2701. doi: 10.3390/ijms21082701. PMID: 32295040; PMCID: PMC7215461.

39. Gimeno-Martos S, Casao A, Yeste M, Cebrián-Pérez JA, Muiño-Blanco T, Pérez-Pé R. Melatonin reduces cAMP-stimulated capacitation of ram spermatozoa. Reprod Fertil Dev. 2019; 31(2):420-31. doi: 10.1071/RD18087. PMID: 30209004.

40. Casao A, Mendoza N, Pérez-Pé R, Grasa P, Abecia JA, Forcada F, et al. Melatonin prevents capacitation and apoptotic-like changes of ram spermatozoa and increases fertility rate. J Pineal Res. 2010; 48(1):39-46. doi: 10.1111/j.1600-079X.2009.00722.x. Epub 2009 Nov 16. PMID: 19919602.

41. Ashrafi I, Kohram H, Ardabili FF. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim Reprod Sci. 2013; 139(1-4):25-30. doi: 10.1016/j.anireprosci.2013.03.016. Epub 2013 Apr 13. PMID: 23664651.

42. Gwayi N, Bernard RT. The effects of melatonin on sperm motility in vitro in Wistar rats. Andrologia. 2002; 34(6):391-6. doi: 10.1046/j.1439-0272.2002.00522.x. PMID: 12472624.

43. Radogna F, Sestili P, Martinelli C, Paolillo M, Paternoster L, Albertini MC, et al.. Lipoxygenase-mediated pro-radical effect of melatonin via stimulation of arachidonic acid metabolism. Toxicol Appl Pharmacol. 2009; 238(2):170-7. doi: 10.1016/j.taap.2009.05.011. Epub 2009 May 19. PMID: 19460395.

44. Zhu Z, Li R, Lv Y, Zeng W. Melatonin protects rabbit spermatozoa from cryo-damage via decreasing oxidative stress. Cryobiology. 2019; 88:1-8. doi: 10.1016/j.cryobiol.2019.04.009. Epub 2019 Apr 26. PMID: 31034812.

45. Ealy AD, Wooldridge LK, McCoski SR. BOARD INVITED REVIEW: Post-transfer consequences of in vitro-produced embryos in cattle. J Anim Sci. 2019; 97(6):2555-68. doi: 10.1093/jas/skz116. PMID: 30968113; PMCID: PMC6541818.

46. Carlomagno G, Minini M, Tilotta M, Unfer V. From Implantation to Birth: Insight into Molecular Melatonin Functions. Int J Mol Sci. 2018; 19(9):2802. doi: 10.3390/ijms19092802. PMID: 30227688; PMCID: PMC6164374.

47. Madan P, Calder MD, Watson AJ. Mitogen-activated protein kinase (MAPK) blockade of bovine preimplantation embryogenesis requires inhibition of both p38 and extracellular signal-regulated kinase (ERK) pathways. Reproduction. 2005; 130(1):41-51. doi: 10.1530/rep.1.00554. PMID: 15985630.

48. Negrón-Pérez VM, Hansen PJ. Role of yes-associated protein 1, angiomotin, and mitogen-activated kinase kinase 1/2 in development of the bovine blastocyst. Biol Reprod. 2018; 98(2):170-83. doi: 10.1093/biolre/iox172. PMID: 29228123; PMCID: PMC6669417.

49. Kuijk EW, van Tol LT, Van de Velde H, Wubbolts R, Welling M, Geijsen N, et al. The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development. 2012; 139(5):871-882. doi: 10.1242/dev.071688. Epub 2012 Jan 25. PMID: 22278923; PMCID: PMC3274353.

50. Kuijk EW, Du Puy L, Van Tol HT, Oei CH, Haagsman HP, Colenbrander B, et al.. Differences in early lineage segregation between mammals. Dev Dyn. 2008; 237(4):918-27. doi: 10.1002/dvdy.21480. PMID: 18330925.

51. Simmet K, Zakhartchenko V, Philippou-Massier J, Blum H, Klymiuk N, Wolf E. OCT4/POU5F1 is required for NANOG expression in bovine blastocysts. Proc Natl Acad Sci U S A. 2018; 115(11):2770-5. doi: 10.1073/pnas.1718833115. Epub 2018 Feb 26. PMID: 29483258; PMCID: PMC5856541.

52. Sakurai N, Takahashi K, Emura N, Fujii T, Hirayama H, Kageyama S, et al. The Necessity of OCT-4 and CDX2 for Early Development and Gene Expression

Involved in Differentiation of Inner Cell Mass and Trophectoderm Lineages in Bovine Embryos. Cell Reprogram. 2016; 18(5):309-18. doi: 10.1089/cell.2015.0081. Epub 2016 Aug 8. PMID: 27500421.

53. Nadeau V, Charron J. Essential role of the ERK/MAPK pathway in blood-placental barrier formation. Development. 2014; 141(14):2825-37. doi: 10.1242/dev.107409. Epub 2014 Jun 19. PMID: 24948605.

54. Hambruch N, Haeger JD, Dilly M, Pfarrer C. EGF stimulates proliferation in the bovine placental trophoblast cell line F3 via Ras and MAPK. Placenta. 2010; 31(1):67-74. doi: 10.1016/j.placenta.2009.10.011. Epub 2009 Nov 14. PMID: 19914712.

55. Alves MBR, de Arruda RP, De Bem THC, Florez-Rodriguez SA, Sá Filho MF, Belleannée C, et al. Sperm-borne miR-216b modulates cell proliferation during early embryo development via K-RAS. Sci Rep. 2019; 9(1):10358. doi: 10.1038/s41598-019-46775-8. PMID: 31316130; PMCID: PMC6637201.

56. Nilendu P, Arumugam K, Mohua DG, Pradeep N, Pushpa RG, Channareddi K, et al., Transcriptomic Profiling of Buffalo Spermatozoa Reveals Dysregulation of Functionally Relevant mRNAs in Low-Fertile Bulls. Front. Vet. Sci., 2021; doi: doi.org/10.3389/fvets.2020.609518.

57. Espino J, Ortiz Á, Bejarano I, Lozano GM, Monllor F, García JF, et al. Melatonin protects human spermatozoa from apoptosis via melatonin receptor- and extracellular signal-regulated kinase-mediated pathways. Fertil Steril. 2011; 95(7):2290-6. doi: 10.1016/j.fertnstert.2011.03.063. Epub 2011 Apr 16. PMID: 21497337.

58. Li MW, Mruk DD, Cheng CY. Mitogen-activated protein kinases in male reproductive function. Trends Mol Med. 2009; 15(4):159-68. doi:

10.1016/j.molmed.2009.02.002. Epub 2009 Mar 18. PMID: 19303360; PMCID: PMC2804913.

Table 1. Primers sequences used for the real-time qPCR

Gene	Primer sequences: (5´-3´)	Fragment size/bp	Accession no.
CDH1	F: GACACCCGGGACAATGTGTA R: GCCCCTATGTAACTGGCTCAA	86	NM_001002763
CDX2	F: CAGAGAGGCAGGTTAAAATTTGGT R: CTGCTGTTGCAACTTCTTCTTGTT	80	NM_001206299.1
MAPK13	F: GAAAACGTCATCGGGCTTCTG R: AGCTGAGTGGATGTACTTAAGACCC	189	NM_001014947.1
AQP3	F: CTGGGCGCTGGAATTATCTTC R: GCCCGAAACAATAAGCTGGTT	78	NM_001079794
BAX	F: GGCTGGACATTGGACTTCCTTC R: TGGTCACTGTCTGCCATGTGG	112	NM_173894.1
HSPB1	F: CTGGACGTCAACCACTTC R: GGACAGAGAGGAGGAGAC	180	NM_001025569.1
POU5F1	F: AGAAGCTGGAGCCGAACC R: CTGCTTTAGGAGCTTGGCAAA	85	NM_174580.3
NANOG	F: TCAGCTACAAGCAGGTGAAGAC R: GCATGCCATTGCTATTCCTC	96	NM_001025344.1
GAPDH	CCCACTCCCAACGTGTCTGT CCTGCTTCACCACCTTCTTGAT	89	NM_001034034.2
Rabbit Globin	F: TACTTCCCCCACTTCGACTTCA R: AGGGCTTCGGACACCTTCTT	74	NM_001082389.2

F: forward; R: reverse.

Table 2. Effects of melatonin addition to the IVF protocol on sperm motion parameters assessed via CASA (LS mean ± SEM).

VARIABLE	Control	Sham-Control	MT-Sperm
Total motility (%)	64.4±1.8[b]	67.9±1.4[ab]	68.4±1.3[a]
Progressive motility (%)	62.8±1.7[b]	66.3±1.4[ab]	66.9±1.3[a]
VAP (μm/s)	113.7±4.4	117.4±2.5	117.9±2.7
VSL (μm/s)	99.6±3.3[b#]	105.4±2.5[ab#]	106.1±2.6[a]
VCL (μm/s)	165.3±4.6[b]	175.0±3.0[a]	176.2±3.3[a]
ALH (μm)	6.1±0.2	6.5±0.2	6.4±0.1
BCF (Hz)	25.3±1.0	26.3±0.8	26.4±0.8
STR (%)	66.1±2.6	68.8±2.2	69.1±2.2
LIN (%)	45.3±2.1	47.1±1.9	47.3±1.9
WOB (%)	50.5±2.2	52.5±1.9	52.6±2.0

Values are LS means ± SEM based on semen samples from 4 different bulls and three replicates each. [a,b] means within rows with different superscripts differ ($P < 0.05$; Tukey HSD Multiple Pairwise Comparisons). [#] $P = 0.054$. Total motility (%): percentage of moving sperm in the entire sample. Progressive motility (%): percentage of sperm that are swimming in a mostly straight line or huge circles. VAP: Average Path Velocity; VSL: Straight Line Velocity; VCL: Curvilinear Velocity; ALH: Amplitude of Lateral Head Displacement; BCF: Beat Cross Frequency; LIN: Linearity; STR: Straightness; WOB: Wobble. Control: without any supplements. Sham-Control: Ethanol in the sperm preparation medium. MT-Sperm: Melatonin in the sperm preparation medium.

Table 3. Effect of incubation time (all experimental groups combined) on sperm motion parameters assessed via CASA after 30-min, 120-min, and 180-min incubation in TALP medium (LS mean ± SEM).

VARIABLE	30-min	120-min	180-min
Total motility (%)	73.1±0.9[a]	64.5±1.3[b]	63.0±1.7[b]
Progressive motility (%)	71.2±0.9[a]	63.1±1.3[b]	61.7±1.7[a]
VAP (μm/s)	113.0±2.3	120.0±3.9	116.2±3.5
VSL (μm/s)	98.6±2.1[b]	105.9±2.8[a]	106.6±3.4[a]
VCL (μm/s)	179.1±3.0[a]	172.5±3.6[ab]	164.9±4.3[b]
ALH (μm)	7.0±0.1[a]	6.2±0.1[b]	5.8±2.2[c]
BCF (Hz)	28.3±0.8[a]	25.4±0.9[b]	24.3±0.7[b]
STR (%)	69.4±1.9	67.1±2.5	67.5±2.7
LIN (%)	45.2±1.5[b]	46.4±2.1[ab]	48.3±2.2[a]
WOB (%)	51.6 ±1.6	51.3±2.2	52.7±2.3

Values are LS means ± SEM based on semen samples from 4 different bulls and three replicates each. [a,b,c] means within rows with different superscripts differ ($P < 0.05$; Tukey HSD Multiple Pairwise Comparisons). Total motility (%): percentage of moving sperm in the entire sample. Progressive motility (%): percentage of sperm that are swimming in a mostly straight line or huge circles. VAP: Average Path Velocity; VSL: Straight Line Velocity; VCL: Curvilinear Velocity; ALH: Amplitude of Lateral Head Displacement; BCF: Beat Cross Frequency; LIN: Linearity; STR: Straightness; WOB: Wobble.

Table 4. Effect of melatonin addition to the IVF protocol on overall sperm quality and function assessed by flow cytometry (LS mean ± SEM).

VARIABLE	Control	Sham-Control	MT-Sperm
Viable (%)	51.1±1.4	51.5±1.7	53.1±1.5
Viable, acrosome intact (%)	39.8±1.6	40.6±1.8	42.3±1.7
Viable, acrosome reacted (%)	11.3±0.4	10.8±0.4	10.9±0.4
Viable, high MMP (%)	61.4±2.0	61.2±1.9	59.5±2.0

Values are LS means ± SEM based on semen samples from 4 different bulls and three replicates each for semen assessment. Values from different incubation times, i.e. 30 min, 120 min, and 180 minutes have been combined according to the experimental groups. Control: without any supplements. Sham-Control: Ethanol in the sperm preparation medium. MT-Sperm: Melatonin in the sperm preparation medium. Viability and acrosome integrity were evaluated with a combined staining of Sybr14, propidium iodide (PI), and PNA-Alexa Fluor 647 (PNA-AF647). Viable sperm (Sybr14 positive/PI negative) were discriminated in acrosome intact (PNA-AF647 negative) and acrosome defect/reacted spermatozoa (PNA-AF647 positive). The percentage of viable sperm with a high mitochondrial transmembrane potential (high MMP) was determined with a combined staining with Sybr14, PI and MitoTracker Deep Red. No statistically significant differences with respect to the factors treatment or incubation time were observed ($P > 0.05$).

Table 5. Effect of melatonin addition to the IVF protocol on *in vitro* fertilization and polyspermy rates.

Treatment Group	Zygotes (*n*)	(+) Fertilization *n* (%)	Polyspermy *n* (%)
Control	24	16 (66.6)[b]	4 (16.6)[a]
Sham-Control	30	15 (50.0)[c]	7 (23.3)[a]
MT-Sperm	29	24 (82.8)[a]	2 (6.9)[b]
MT-Co-incubation	32	23 (71.9)[ab]	3 (9.4)[b]

[a,b,c] Chi-Square test: Proportions within columns with different superscripts differ ($P < 0.01$). (+) Fertilization refers to normal-fertilized zygotes with the presence of two pronuclei. Polyspermy refers to those zygotes with at least three or more pronuclei identified. Control: without any supplements. Sham-Control: Ethanol in the sperm preparation medium. MT-Sperm: Melatonin in the sperm preparation medium. MT-Co-incubation: melatonin in the *in vitro* fertilization co-incubation medium. Data collected from three IVF replicates.

Table 6. Effect of melatonin addition to the IVF protocol on *in vitro* embryo developmental rates.

Treatment Group	Zygotes *n*	Cleavage *n* (%)	Blastocysts *n* (%)	Advanced bl. *n* (%)
Control	156	115 (73.7)	45 (39.1)[b]	24 (20.9)[b]
Sham-Control	147	118 (80.3)	54 (45.8)[ab]	35 (29.7)[ab]
MT-Sperm	145	115 (79.3)	63 (54.8)[a]	38 (33.0)[a]
MT-Co-incubation	152	121 (79.6)	58 (47.9)[ab]	33 (27.3)[ab]
Average	600	469/600 (78.2)	220/469 (46.9)	130/469 (27.7)
Summary statistics (logistic procedure)				
P value	-	NS	0.0179	0.04
OR	-	-	1.885	1.871
95% CI	-	-	1.115-3.184	1.033-3.390

[a,b] Logistic procedure: Percentages within columns with different superscripts differ ($P < 0.05$). Blastocyst rate is considered as the proportion of cleaved zygotes that reached the blastocyst stage. Advanced bl. refers to advanced blastocysts (expanded and hatching) based on the number of cleaved zygotes. OR: Odds ratio. 95% CI: 95% confidence intervals limits for relative risks. Control (n: 156): without any supplements. Sham-Control (n: 147): Ethanol in the sperm preparation medium. MT-Sperm (n: 145): Melatonin in the sperm preparation medium. MT-Co-incubation (n: 152): melatonin in the *in vitro* fertilization co-incubation medium. Cleavage: NS: no significant ($P > 0.05$). Data collected from six IVF replicates.

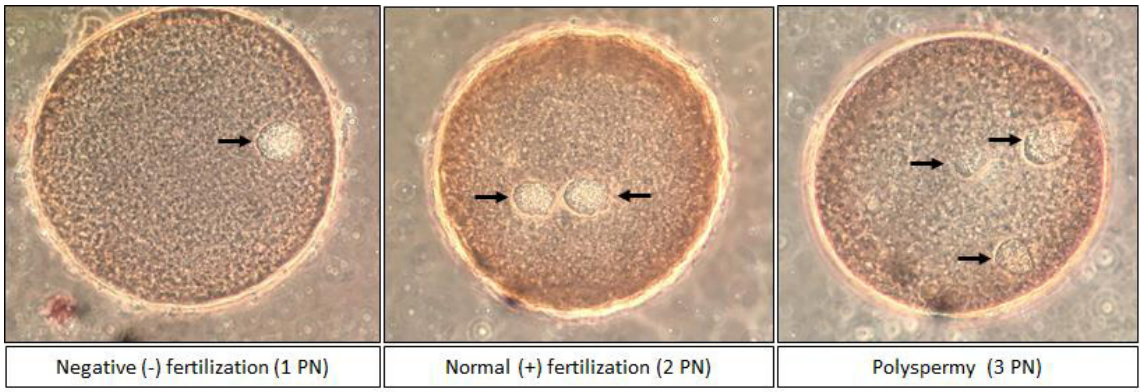

Fig 1. Pronuclear (PN) assessment and determination of fertilization via Lacmoid staining to evaluate the *in vitro* fertilization rate (arrows indicate the pronuclei).

A)

Cluster	VCL (μm/s)	ALH (μm/s)	BCF (Hz)	LIN (%)	% sperm
1	140.6±31.6	3.8±1.6	43.2±5.8	75.3±8.8	9.0
2	248.0±28.7	7.8±1.8	34.5±7.1	67.9±7.0	47.5
3	280.5±31.6	11.4±2.3	33.8±6.7	50.4±7.2	16.2
4	187.7±35.4	8.6±2.5	30.5±5.4	56.6±9.0	6.3
5	263.0±35.8	12.3±3.0	29.8±7.5	29.8±7.5	3.9
Rest					17.1

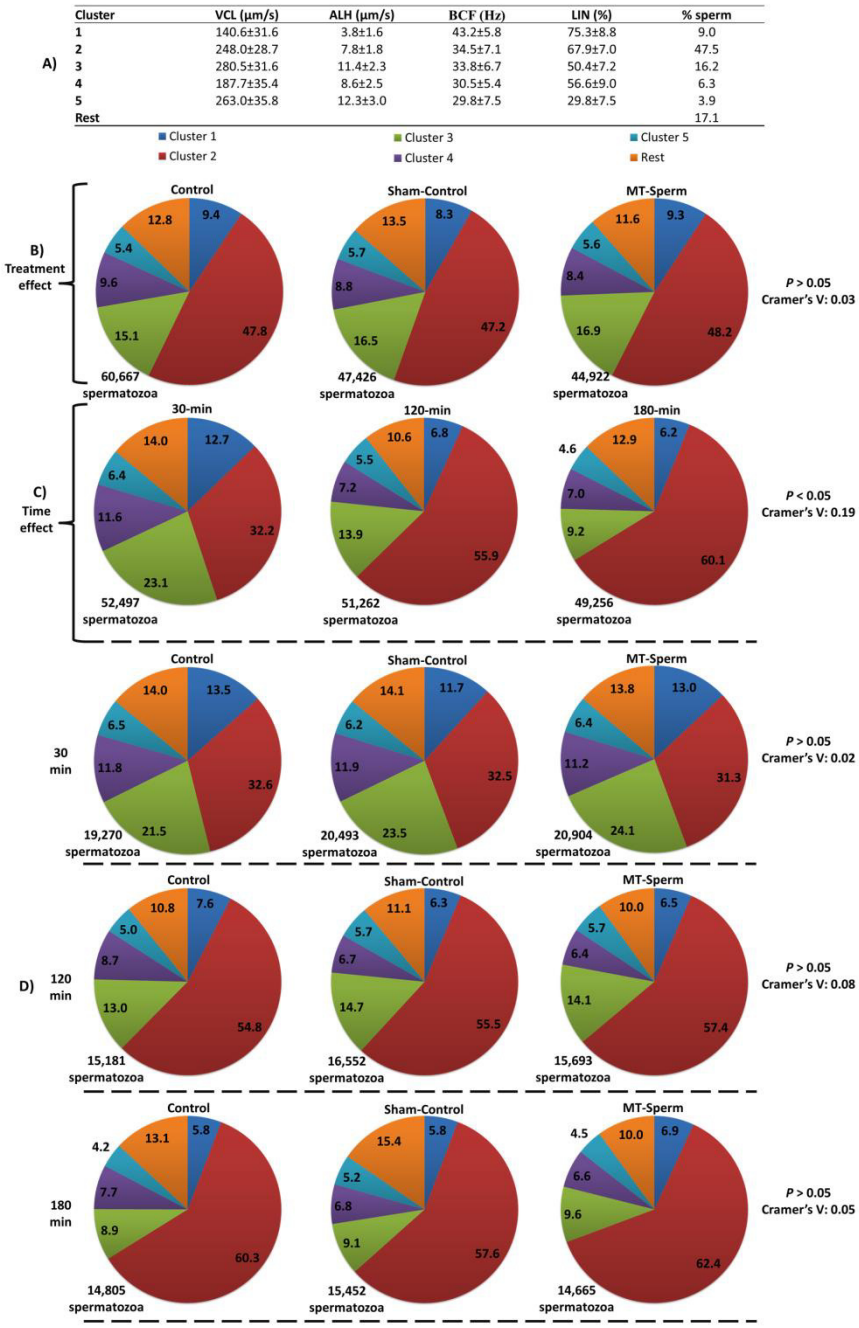

Fig 2. Cluster analysis of sperm kinematic parameters. Distribution of bovine sperm subpopulations treated with or without melatonin during the post-thaw sperm preparation protocol for IVF after different incubation periods. Treatment groups: Control: standard TALP modified-medium, Sham-control: group containing ethanol in the TALP modified-medium, and MT-Sperm: melatonin added to the TALP modified-

medium; times of incubation in TALP medium (30 min, 120 min, and 180 min). The analysis was based on a total of 153,015 sperm tracks from 108 samples recorded after 30, 120 and 180 minutes of incubation period. Only clusters containing more than 5% spermatozoa are displayed. A) Motility descriptors (mean ± S.D.) are given for each cluster as well as the percentage of sperm assigned to each of them. Distribution of spermatozoa to different clusters based on motility parameters to the different treatment groups (B, all time points combined), incubation period (C, all treatments combined) and a stratified view of the dataset (D). Sperm distribution to the different clusters changed significantly depending on the incubation time ($P < 0.05$). Cramer's V (ranging from 0-1) indicates the effect size that incubation or the treatment had on the distribution of sperm to the different clusters, i.e., the size of sperm subpopulations with distinct motility patterns. For interpretation, the following definitions were used: $V < 0.10$ = no effect, $0.10 < V \leq 0.30$ = slight effect, $0.30 < V \leq 0.50$ = moderate effect, $V > 0.50$ = strong effect.

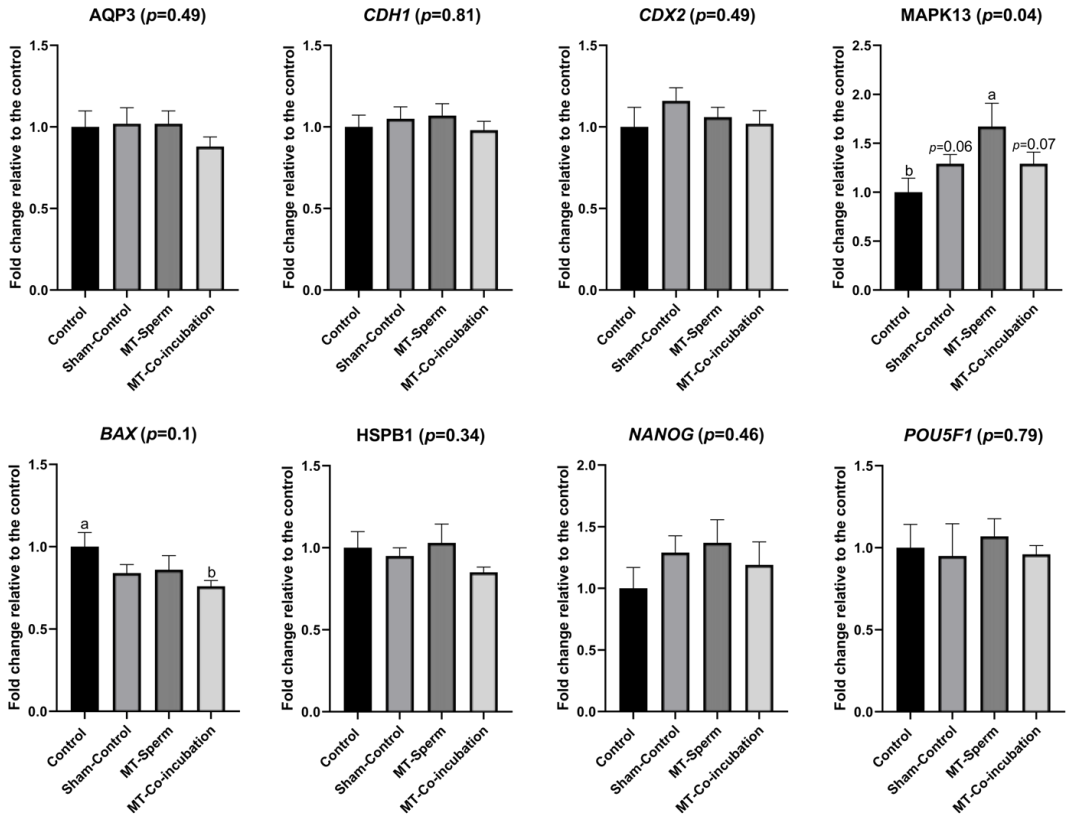

Fig 3. **Relative mRNA abundance of genes related to embryo developmental pathways in expanded blastocysts derived from sperm treated or not with melatonin in the IVF protocol.** Cadherin 1 (*CDH1*), caudal type homeobox 2 (*CDX2*), the regulator of MAP kinase activity (MAPK13), water transport gene aquaporin 3 (*AQP3*)], apoptosis regulation [BCL2 Associated X, Apoptosis Regulator (*BAX*), and Heat shock protein beta-1 (*HSPB1*)], and pluripotency [POU domain, class 5, transcription factor 1 (*POU5F1*), and the Nanog Homeobox (*NANOG*)] in the in vitro-derived expanded blastocysts. Data are represented as fold change relative to the control after normalizing the geometric mean from the housekeeping genes (Globin/Gapdh) and are means ± SEM. Different letters (a-b) indicate significant differences amongst experimental groups ($p<0.05$). Percentages with p values above the SEM show statistical trends, whereas p values enclosed within parenthesis refer to significance level found in the statistical model for each evaluated gene.

Supporting information

S1 Table. Parameter Settings CASA IVOS (Version 12.0 IVOS Hamilton Thorne Bioscience, Beverly, USA).

(DOCX)

S2 Table. Flow cytometry settings Flow cytometer settings (Gallios™, Beckman Coulter, Germany).

(DOCX)

S3 Table. Sperm quality and functionality parameters observed in the evaluated bulls.

(DOCX)

S4 Table. Effect of melatonin addition to the IVF protocol on freezing/thawed sperm motion parameters assessed through CASA after 30-min, 120-min, and 180-min incubation.

(DOCX)

S5. Effect of melatonin addition to the IVF protocol on freezing/thawed sperm motion functionality assessed through FACS after 30-min, 120-min, and 180-min of the incubation period.

(DOCX)

Supporting Table 1. Parameter Settings CASA IVOS (Version 12.0 IVOS Hamilton Thorne Bioscience, Beverly, USA).

Analysis Setup	Setting
Apply sort	0
Frames acquired	60
Frame rate	60 Hz
Minimum contrast	40
Minimum cell size	5 pixels
Minimum static contrast	15
Straightness (STR) Threshold	25%
VAP cutoff	5.0 μm/s
Progressive minimum VAP	10.0 μm/s
VSL cutoff	5 μm/s
Cell intensity	70
Static head size	0.26 to 7.88
Static head intensity	0.14 to 1.99
Static elongation	10 to 98
Slow cells motile	No
Magnification	8.75
Magnification	8.75
Video frequency	60
Bright field	No
LED illumination intensity	2188
IDENT illumination intensity	2598
Temperature, Set	37.5 °C
Chamber depth	10 μm
Chamber position	14.5 μm
Chamber position B	15.5 μm
Chamber position C	16.5 μm
Chamber position D	17.5 μm
Chamber type	Makler
Field selection mode	Auto
IDENT fluorescent mode	OFF
Integrating time	1 Frame

Supporting Table 2. Flow cytometer settings (Gallios™, Beckman Coulter) used for sperm stained for plasma membrane integrity with Sybr14/Propidium Iodide (PI) (LIVE/DEAD®Sperm Viability Kit), in combination with either acrosome integrity using peanut agglutinin (PNA-Alexa Fluor™ 647) or mitochondrial membrane potential (Mito Tracker™ Deep red FM). Abbreviations: FS: forward scatter, SS: side scatter, FL1: fluorescence channel 1, FL3: fluorescence channel 3, FL6: fluorescence channel 6.

Sybr14/PI/PNA-Alexa Fluor™ 647 (PNA-AF647)					
Setting	**FS**	**SS**	**FL1**	**FL3**	**FL6**
Voltage	124	390	688	421	730
Gain	2.0	10	1.0	1.0	1.0
Discriminator	120	Off	Off	Off	Off
Sybr14/PI/Mito Tracker™ Deep red FM (Deep Red FM)					
Setting	**FS**	**SS**	**FL1**	**FL3**	**FL6**
Voltage	124	213	704	371	791
Gain	2.0	10	1.0	1.0	1.0
Discriminator	120	Off	Off	Off	Off
Parameter Settings					
Compensation factor (FL1 to FL3)			Sybr14/PI/PNA-AF647		14.6
			Sybr14/PI/Deep red FM		20.9
Compensation factor (FL3 to FL1)			Sybr14/PI/PNA-AF647		0.4
			Sybr14/PI/Deep red FM		0.1
Compensation factor (FL3 to FL6)			Sybr14/PI/PNA-AF647		0.0
			Sybr14/PI/Deep red FM		0.0
Compensation factor (FL6 to FL3)			Sybr14/PI/PNA-AF647		2.4
			Sybr14/PI/Deep red FM		4.5
Blue laser			On, Shutter Closed		
Excitation wavelength (nm) Sybr14			488		
Excitation wavelength (nm) PI			488		
Excitation wavelength (nm) PNA-AF647			633		
Excitation wavelength (nm) Deep red FM			633		
Detection wavelength FL1:			525 nm: 40 band pass		
Detection wavelength FL3:			620 nm: 30 band pass		
Detection wavelength FL6:			660 nm: 20 band pass		
Sybr14 detection channel			FL1		
PI detection channel			FL3		
PNA-AF647 detection channel			FL6		
Deep red FM detection channel			FL6		
Software version			Gallios 1.2		

Supporting Table 3. Sperm quality and functionality parameters observed in the evaluated bulls.

	Bull			
Variable	**1**	**2**	**3**	**4**
Total motility (%)	73.9±2.0	73.2±1.6	74.3±1.6	71.0±2.0
Progressive motility (%)	72.0±6.0	71.3±4.6	72.1±1.5	69.2±1.9
Viable (%)	65.6±1.2^{a}	60.9±2.5^{ab}	61.9±1.2^{ab}	54.9±2.1^{b}
Viable, acrosome intact (%)	56.5±1.7^{a}	48.8±3.1^{a}	52.3±1.5^{ab}	42.5±2.1^{b}
Viable, high MMP (%)	64.4±0.8^{b}	56.5±2.5^{c}	62.7±1.2^{bc}	77.7±1.9^{a}

Values are the means ± SEM. ^{a,b} means among rows with different superscripts differ ($P < 0.05$); Tukey HSD Multiple Pairwise Comparisons). Total motility (%): percentage of moving sperm in the entire sample. Progressive motility (%): percentage of sperm swimming in a mostly straight line or huge circles. Viability and acrosome integrity were evaluated with a combined staining of Sybr14, propidium iodide (PI), and PNA-Alexa Fluor 647 (PNA-AF647). Viable sperm (Sybr14 positive/PI negative) were discriminated in acrosome intact (PNA-AF647 negative) and acrosome defect/reacted spermatozoa (PNA-AF647 positive). The percentage of viable sperm with a high mitochondrial transmembrane potential (high MMP) was determined with a combined staining with Sybr14, PI and MitoTracker Deep Red FM. Bull number 4 was selected for the IVF trial.

Supporting Table 4. Effect of melatonin addition within the IVF protocol on freezing/thawed sperm motion parameters assessed through CASA after 30-min, 120-min, and 180-min incubation.

	Control			Sham-Control			MT-Sperm		
Variable	**30-min**	**120-min**	**180-min**	**30-min**	**120-min**	**180-min**	**30-min**	**120-min**	**180-min**
Total motility (%)	70.9±3.3^{abc}	61.6±3.3^{d}	60.5±3.3^{d}	73.2±3.3^{ab}	67.6±3.3^{abcd}	62.8±3.3^{cd}	75.2±3.3^{a}	64.3±3.3^{bcd}	65.7±3.3^{bcd}
Progressive motility (%)	69.0±3.2^{abc}	60.1±3.2^{cd}	59.3±3.2^{d}	71.3±3.2^{ab}	66.2±3.2^{abcd}	61.4±3.2^{cd}	73.2±3.2^{a}	62.9±3.2^{bcd}	64.5±3.2^{abcd}
VAP (µm/s)	109.4±8.4	120.9±8.4	110.7±8.4	113.4±8.4	121.4±8.4	117.4±8.4	116.2±8.4	117.8±8.4	120.3±8.4
VSL (µm/s)	95.5±7.8^{b}	102.1±7.8^{ab}	101.3±7.8^{ab}	99.2±7.8^{ab}	109.4±7.8^{ab}	107.6±7.8^{ab}	101.2±7.8^{ab}	106.2±7.8^{ab}	110.9±7.8^{a}
VCL (µm/s)	173.2±9.4^{ab}	165.8±9.4^{ab}	156.8±9.4^{b}	179.4±9.4^{a}	179.2±9.4^{a}	166.6±9.4^{ab}	184.6±9.4^{a}	172.6±9.4^{ab}	171.4±9.4^{ab}
ALH (µm)	6.8±0.4^{abc}	6.0±0.4^{cde}	5.5±0.4^{c}	7.0±0.4^{ab}	6.5±0.4^{abcd}	5.9±0.1^{cd}	7.3±0.4^{a}	6.2±0.4^{bcde}	5.2±0.4^{cde}
BCF (Hz)	27.4±2.6^{abc}	25.0±2.6^{cd}	23.6±2.6^{d}	28.2±2.6^{ab}	26.1±2.6^{bcd}	24.6±2.6^{cd}	29.2±2.6^{a}	25.1±2.6^{cd}	24.5±2.6^{cd}
LIN (%)	44.1±6.2	45.4±6.2	46.5±6.2	45.2±6.2	47.7±6.2	48.5±6.2	46.2±6.2	46.1±6.2	49.7±6.2
STR (%)	67.6±7.6	65.7±7.6	65.1±7.6	69.4±7.6	69.0±7.6	67.9±7.6	71.1±7.6	66.6±7.6	69.5±7.6
WOB (%)	50.3±6.6	50.1±6.6	51.0±6.6	51.5±6.6	52.9±6.6	53.1±6.6	52.9±6.6	50.9±6.6	54.0±6.6

Values are the means ± SEM. ^{a,b,c,d} means among rows with different superscripts differ ($P < 0.05$); Tukey HSD Multiple Pairwise Comparisons). Total motility (%): percentage of moving sperm in the entire sample. Progressive motility (%): percentage of sperm that are swimming in a mostly straight line or huge circles. VAP: Average Path Velocity; VSL: Straight Line Velocity; VCL: Curvilinear Velocity; DAP: Distance Average Path; DSL: Distance Straight Line; DCL: Distance curvilinear; ALH: Amplitude of Lateral Head Displacement; BCF: Beat Cross Frequency; LIN: Linearity; STR: Straightness; WOB: Wobble. Control: without any supplements. Sham-Control: Ethanol in the sperm preparation medium. MT: Melatonin in the sperm preparation medium. Warning: The interaction treatment by incubation period within experimental groups was not significant in the overall statistical model ($P > 0.05$).

Supporting Table 5. Effect of melatonin addition within the IVF protocol on freezing/thawed sperm motion functionality assessed through FACS after 30-min, 120-min, and 180-min of the incubation period.

	Control			Sham-Control			MT-Sperm		
Variable	**0**	**2**	**3**	**0**	**2**	**3**	**0**	**2**	**3**
Viable (%)	58.3±2.0^{a}	48.0±1.8^{b}	47.0±2.0^{b}	61.3±2.1^{a}	49.9±1.8^{b}	43.4±1.8^{b}	62.6±2.3^{a}	48.8±1.5^{b}	48.4±1.8^{b}
Viable, high MMP	64.4±2.9^{ab}	61.2±3.6^{abc}	58.5±4.0^{bc}	65.4±2.9^{ab}	59.8±3.2^{abc}	58.9±3.7^{abc}	67.5±3.0^{a}	57.6±2.8^{bc}	53.5±3.4^{c}
Viable, acrosome intact	47.0±2.4^{ab}	36.7±2.0^{c}	35.8±2.6^{c}	50.4±2.9^{a}	38.7±2.2^{bc}	33.4±2.2^{c}	51.7±2.8^{a}	37.2±1.8^{c}	38.0±2.4^{c}
Viable, acrosome reacted	11.3±0.7	11.3±0.7	11.2±0.9	11.1±0.7	11.2±0.6	10.0±0.9	10.9±0.6	11.3±0.5	10.4±0.9

Values are the means ± SEM. ^{a,b,c} means among rows with different superscripts differ ($P < 0.05$); Tukey HSD Multiple Pairwise Comparisons). Values are the means ± SEM. Viability and acrosome integrity were evaluated with a combined staining of Sybr14, propidium iodide (PI), and PNA-Alexa Fluor 647 (PNA-AF647). Viable sperm (Sybr14 positive/PI negative) were discriminated in acrosome intact (PNA-AF647 negative) and acrosome defect/reacted spermatozoa (PNA-AF647 positive). The percentage of viable sperm with a high mitochondrial transmembrane potential (high MMP) was determined with a combined staining with Sybr14, PI and MitoTracker Deep Red FM. Control: without any supplements. Sham-Control: Ethanol in the sperm preparation medium. MT: Melatonin in the sperm preparation medium. Warning: The interaction treatment by incubation period within experimental groups was not significant in the overall statistical model ($P > 0.05$).

Author contributions

Juan Carlos Gutiérrez-Añez: Conceptualization: conceived and designed the experiments, Investigation: performed the IVF trial, data acquisition, Formal analysis: contribute with the statistical analysis, Writing, and original draft preparation. Heiko Henning: Validation, Formal analysis: contribute with the statistical analysis, Writing. Andrea Lucas-Hahn: Conceptualization: contribute with the experimental design, Validation, Writing - review & editing, Resources. Ulrich Baulain: Formal Analysis: application of the statistical and mathematical approach to analyze the data. Patrick Aldag: Investigation: performed the IVF trial. Birgit Sieg: Investigation: performed the flow cytometry trial and contributed to the data analysis. Vivian Hensel: Investigation: performed de Computer Assisted Sperm Analysis (CASA), contribute to the interpretation of the data. Doris Herrmann: designed the gene expression analysis, performed de RT-qPCR trial, and contribute to the interpretation of the data. Heiner Niemann: Conceptualization, Supervision, Validation, Writing - review & editing, Resources, and the final version's approval.

Chapter III

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Melatonin enhances *in vitro* developmental competence of cumulus-oocyte complexes collected by ovum pick-up in prepubertal and adult dairy cattle

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Analysis: JCGA

Scientific writing: JCGA, HN

Abstract

Bovine oocytes from prepubertal donors have been used for *in vitro* embryo production to decrease the generation interval. However, reduced cumulus-oocyte competence, mainly attributed to increased apoptosis, has been observed in oocytes/embryos collected from prepubertal donors. Here, we investigated the effects of the potent antioxidative molecule melatonin on cumulus-oocyte competence and embryo development in prepubertal and adult dairy cattle *in vitro*. A total of fifteen Holstein Friesian calves, six to ten months old (7.6 ± 1.34 months of age) and fifteen adult cows with one to four calvings (2.3 ± 0.96 calvings) were enrolled as ovum pick up (OPU) donors in this study. Cumulus-oocyte complexes (COCs) were cultured either in the presence or absence of melatonin (0.01 nM). The proportion of cleavage stages, blastocysts, and advanced blastocysts was determined. Embryo quality was assessed via differential staining to determine the total embryonic cells and allocation to the inner cell mass (ICM) and trophectoderm (TE) cells. Melatonin treatment yielded a greater percentage of blastocysts compared to the control group, i.e. oocytes from both adult cows (P = 0.0485; 24.8 ± 3.5% vs. 16.0 ± 3.4%, respectively), and from prepubertal donors (P = 0.0007; Melatonin 23.1 ± 5.1% vs. Control: 11.1 ± 3.5%). Adult cows had significantly (P = 0.0370) greater advanced blastocyst rates than those found in the prepubertal group (13.9%± vs. 7.0±%, respectively). Additionally, the number of ICM, total cells, and the ratios ICM: Total, ICM: TE, respectively, were greater (P < 0.05) after melatonin treatment compared with the control group (39.1 ± 2.8, 98.6 ± 5.7, 0.4 ± 0.01, and 0.7 ± 0.04 vs. 27.3 ± 2.9, 81.2 ± 5.8, 0.34 ± 0.01, and 0.52 ± 0.04, respectively). Blastocysts derived from adult cows had a greater number of TE (P < 0.01) and total embryonic cells (P = 0.0095) compared to the prepubertal donor group (63.5 ± 3.2 and 101.05 ± 4.8 vs. 48.9 ± 4.3 and 78.8 ± 6.5, respectively). Nevertheless, embryonic cell counting in embryos derived from prepubertal COCs equated to that observed from adult donors after melatonin exposure. In conclusion, these results indicate that the presence of melatonin during *in vitro* maturation improves cumulus-oocyte competence, embryo development, and quality by increasing the allocation of embryonic cells to the ICM compartment and the total number of embryonic cells in both adult and prepubertal bovine donors.

Chapter IV

4 Manuscript 3

Melatonin supplementation during *in vitro* production of bovine embryos: Implications for gene expression and preimplantation embryo competence

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Data collection: JCGA, PA, DH

Analysis: JCGA

Scientific writing: JCGA, HN

Abstract

Melatonin is a radical scavenger and is thought to have anti-oxidative functions in early mammalian development. Here, we investigated the effects of melatonin (MT) (1.0 nM and 0.01 nM) on *in vitro* embryo development by determining the number of morulae, blastocysts, and advanced blastocysts on days 6, 7, 7.5, respectively, counting the number and type of embryonic cells (ICM or TE) and analyzing a panel of marker genes, indicative for developmental competence (*CDH1*, *CDX2*, *MAPK13*), and ion exchange and water transport (*AQP3*, *ATP1A1*, *ATP1B1*). As melatonin had to be dissolved in ethanol, a Sham control and a standard Control group were included in the study. The MT 0.01 nM group had significantly (p<0.01) greater embryo developmental rates compared to the Control and Sham control groups (53.8.6% vs. 45.9% and 46.0%, respectively). Melatonin supplementation increased (p<0.05) the total number of cells and specifically of trophectoderm cells compared to controls. Gene expression analysis revealed that expanded blastocysts from day 7.5 derived from the MT 1.0 nM group exhibited increased (p<0.05) expression of *CDH1* and *AQP3* over the Control group. In conclusion, *CDH1* and *AQP3* are likely involved in melatonin mediated regulation of oocyte competence and embryo development *in vitro*.

Additional keywords: antioxidant, differential staining, embryonic cell counting, ethanol, *in vitro* fertilization, IVF.

Introduction

The potential of melatonin to prevent oxidative damage and to counteract the production of reactive oxygen species (ROS) usually encountered under *in vitro* conditions has been evaluated in several species. Enhanced oocyte competence and improved *in vitro* embryo development via antioxidative effects by melatonin

supplementation have been observed in humans (Zou et al. 2020; Loren et al. 2017), cattle (Komninou et al. 2016; Zhao et al. 2015), buffalo (Nagina et al. 2016), sheep (Tian et al. 2017), goats (Soto-Heras et al. 2018, 2019), and pigs (Yang et al. 2020; Kim et al. 2019; Wang et al. 2017).

After supplementation of media with melatonin, a variety of positive effects has been observed, including improved cytoplasmic oocyte maturation (Zhao et al. 2018), increased ability for cell division, enhanced cell compaction, blastocyst formation, and hatching capability (Remião et al. 2016; Komninou et al. 2016; Wang et al. 2014), improved oocyte developmental competence in bovine adult and prepubertal donors (Gutiérrez-Añez et al. 2021), and increased embryonic cell counts (Gutiérrez-Añez et al. 2021; Komninou et al. 2016). Furthermore, melatonin was found to compensate suboptimal culture conditions (high oxygen tension level of 20%) for bovine embryos (Papis et al. 2007).

Early mammalian embryogenesis critically depends on a tightly controlled, well-orchestrated program of gene expression. Fertilization and the early cleavage stages are associated with massive epigenetic reprogramming of the paternal and maternal chromatin and initiated by maternal RNA and proteins accumulated during oogenesis and the final stages of oocyte maturation (Schultz, 1993). Large scale synthesis of messenger RNA from the diploid embryonic genome is initiated at a species-specific time point. This occurs in murine embryos at the end of the first cell cycle, in human embryos in four- and eight-cell stages (Braude et al., 1988), and in bovine embryos at the 8-cell stage (Telford et al. 1990). This complex gene expression pattern has frequently been shown to become deviant from the physiological level during *in vitro* embryo production.

One of the compaction-associated transcripts studied is the epithelial cell-adhesion molecule E-cadherin type 1 (*CDH1*), a coding protein essential during morula compaction (Maître et al. 2015), lineage allocation and cell polarity during blastocyst formation (Johnson and McConnel 2004). The active p38 α/β MAPK signaling is primarily required for development from the 8- to 16-cell stage to the blastocyst stage, mainly by regulating filamentous actin during murine preimplantation development (Natale et al. 2004) and blastocyst cavity formation (Maekawa et al. 2005). The caudal-type homeobox 2 (*CDX2*) is essential for early preimplantation embryo development, gene expression, and inner cell mass (ICM) and trophectoderm (TE) lineage differentiation. Bovine *CDX2* downregulation caused delayed blastocyst formation (Sakurai et al. 2016), while its mRNA depletion predisposed loss of TE epithelial layer integrity (Goissis and Cibelli, 2014).

Cavitation (blastocyst formation) is mediated by fluid transfer across the outer blastomeres, eventually forming the fluid-filled cavity (the blastocoel), which is controlled by a family of water transport and ion exchange-related genes, including the aquaporins (*AQP*) gene family (Watson et al. 2004; Sponchiado et al. 2017) and Na+-K+-adenosine triphosphatase (ATPase) activity isoforms localized in the basolateral membranes of the trophectoderm (Gordon 2003; Houghton et al. 2003). Aquaporin molecules play a role in cavitation, allowing water movement across the trophectoderm. Simultaneously, the Na / K ATPase enzyme contributes to establishing and maintaining an ionic gradient across the trophectoderm, contributing to the osmotic accumulation of water during blastocoel formation and embryo expansion (Camargo et al. 2011). Na/K-ATPase isoforms such as the Na/K-ATPase β1 subunit expression and α1β1 isozyme drive fluid transport towards the blastocoel (MacPhee et al. 2000), whereas the β1

subunit is also required for a regular assembly of the trophectoderm (TE) tight junction-associated proteins (Madan et al. 2005; Violette et al. 2006).

Here, we investigated the effects of melatonin on *in vitro* embryo development and monitored developmental timing during *in vitro* development from morulae to expanded blastocysts and determined number and type of embryonic cells (ICM or TE) at defined days of *in vitro* culture. Moreover, we analyzed the mRNA expression of two developmentally-important gene families, incl. (I) genes involved in morula and blastocyst formation, i.e. type 1 epithelial cadherin 1 (*CDH1*), the caudal type homeobox 2 (*CDX2*), and the regulator of MAP kinase activity (*MAPK13*), and (II) genes involved in ion exchange and water transport genes such as aquaporin 3 (*AQP3*), the ATPase Na+/K+ transporting subunits alpha 1 (*ATP1A1*), and beta 1 (*ATP1B1*).

Materials and methods

Reagents, melatonin dissolving system and experimental groups

Unless otherwise stated, all reagents used were purchased from Sigma-Aldrich (Steinheim, Germany). The melatonin (MT) stock solutions were prepared as recently described (Gutiérrez-Añez et al. 2021). Briefly, melatonin stock solutions were prepared in an Ethanol/TCM (tissue culture based-medium 199, Sigma-Aldrich), enriched with 1 mg/ml fatty acid-free bovine serum albumin (FAF-BSA, Sigma-Aldrich) at a concentration of 10^{-7} M and 10^{-9} M, each containing 1% ethanol and were stored in aliquots of 30 µL at -20 °C for no longer than four weeks.

Prior to use in *in vitro* maturation (IVM), fertilization (IVF), and embryo culture (IVC), the aliquots were thawed and diluted 1:100 in each respective medium resulting in two experimental groups of melatonin with a concentration of 10^{-9} M and 10^{-11} M (from now onwards called MT 1.0 nM and MT 0.01 nM), respectively, each containing

0.01% of ethanol. To take possible effects from the vehicle ethanol into account, we set up a sham control group containing only 0.01% ethanol without any melatonin (Sham control) and a control group without any supplement (Control).

Cumulus-oocyte complexes collection and selection

Cumulus-oocyte complexes (COCs) were collected by aspirating follicles (2-6 mm in size) manually from slaughterhouse-derived ovaries with an 18-gauge needle. The needle was attached to a homemade screw cap that had two connectors, one to attach the needle, and the other remained free to allow air entrance. Follicular aspiration was performed by sucking the follicular fluid into a 50 ml Falcon™ tube, containing 5 ml 37 °C TCM medium using atmospheric pressure and soft compression exerted on the ovary during aspiration. The temperature was kept at ~37 °C throughout the entire process.

Cumulus-oocyte complexes (COCs) were selected and graded according to quality. COCs in grade I (homogeneous dark ooplasm with at least three layers of dark and compact cumulus cells), and in grade II (presence of a bright and with at least three layers of compact cumulus cells, and translucent ooplasm) were employed in these experiments.

In vitro maturation (IVM)

For IVM, COCs were pooled in groups of 25-30 and incubated in 250 µL tissue culture based-medium 199 (TCM, Sigma-Aldrich), enriched with 1 mg/ml fatty acid-free bovine serum albumin (FAF-BSA, Sigma-Aldrich), supplemented with 10 IU/ml equine chorionic gonadotropin (eCG) and 5 IU/ml human chorionic gonadotropin (hCG) (Suigonan®, MSD, Intervet, Unterschleissheim, Germany). COCs were incubated

at 38.5 °C and 5% CO_2 in a humidified atmosphere for 24 h in four-well dishes (Nunclon™ Delta Surface, Thermo Scientific, Roskilde, Denmark).

In vitro fertilization (IVF)

After maturation, expanded COCs were placed in 250 µL fertilization medium (Fert-TALP), supplemented with 6 mg/ml of FAF-BSA, ten (10) µM hypotaurine, 1.0 IU/ml heparin, and 1.0 µM epinephrine into the above described four-well dishes. Frozen-thawed sperm from a Holstein bull with proven fertility in IVF from the same batch with a concentration of 20-25 x 10^6 spermatozoa / 0.5-ml was used throughout these experiments. Semen selection and purification were carried out via a modified colloidal gradient-solution protocol (BoviPure™, Nidacon, Mölndal, Sweeden). Briefly, a single semen straw was first thawed in a water bath at 33 °C for 1 min and then passed through two concentrations of the Bovipure solution, one at 40% (200 µl BoviPure™ + 300 µl BoviDilute™), and the other at 80% (400 µl BoviPure™ +100 µl BoviDilute™) in 1.5 ml Eppendorf centrifuge tubes. Prior to use, both colloidal solutions were stabilized for one hour in the incubator at 39 °C and 5 % CO_2. During thawing, the 40% solution was placed over the solution at 80%, and then the semen was placed on this colloidal solution prior to centrifugation.

After centrifugation at 400 *g* at room temperature for nine minutes, 900 µL supernatant from the sample was removed, leaving the sperm pellet in ~100 µL of the mixed colloidal solution. Then, the sample of the purified sperm was washed, eluted, and centrifuged for three min at 400 g with a home-made washing solution containing 500 µl TALP modified-medium enriched with 6 mg/ml BSA, 0.05 mg/ml gentamicin, 0.028 mg/ml Na-pyruvate. All experimental groups were fertilized with the same semen

preparation with a ratio of 100.000 sperm cells/100 µL of IVF medium (total 250.000 sperm cells per well contained 250 µL of IVF medium).

In vitro culture (IVC)

Eighteen to twenty hours (18-20 h) after fertilization, presumptive zygotes were placed into four-well dishes containing 500 µL *in vitro* culture (IVC) medium based on synthetic oviductal fluid (SOF) enriched with 4 mg/ml of BSA-FAF covered with 600 µL mineral oil (GYNEMED, Lensahn, Germany). Zygotes were incubated at 38.5 °C, 5% CO_2, and 5% O_2 in a humidified atmosphere. Unlike IVC, both IVM and IVF system were oil-free. During each *in vitro* embryo production step (IVM, IVF, and IVC), the empty corners of the dishes' surrounding areas were filled with 300 µL bi-distilled water each to complete 1.2 ml per plate at the beginning of each culture step.

In vitro embryo production and experimental design

Embryos from a total of 27 IVF cycles were used for the different experiments. In the first experiment, COCs from six IVF cycles or replicates were used to obtain embryos at the morula stage [day 6, *n*: 1016 zygotes (Control: *n*: 254; Sham control: *n*: 272; MT 1.0 nM: *n*: 211; MT 0.01 nM: *n*: 279)]. In the second experiment, six replicates were performed to produce embryos at the blastocyst stage [day 7, *n*: 646 zygotes (Control: *n*: 158; Sham control: *n*: 164; MT 1.0 nM: *n*: 157; MT 0.01 nM: *n*: 167)]. In the third experiment, ten replicates were completed to produce advanced blastocysts (expanded and hatched blastocysts) [day 7.5, *n*: 996 zygotes (Control: *n*: 247; Sham control: *n*: 249; MT 1.0 nM: *n*: 247; MT 0.01 nM: *n*: 253)]. Likewise, five IVF cycles or replicates were made in the fourth experiment designed to produce day 7.5 advanced blastocysts for embryonic cell counting to evaluate embryo competence. A total of 97 advanced blastocysts [day 7.5, 68 expanded and 29 hatched (Control: *n*: expanded: 17,

hatched: 7; Sham control: *n*: expanded: 20, hatched: 7; MT 1.0 nM: *n*: expanded: 14, hatched: 7; MT 0.01 nM: *n*: expanded: 17, hatched: 8)] were differentially stained to determine the number of ICM and TE cells.

IVF success rates were evaluated by determining the rates of cleavage (Experiments 1, 2, and 3), morulae (Experiment 1), blastocysts, and advanced blastocysts (Experiments 2, 3, and 4). The cleavage rate was assessed at 72 h post-insemination (hpi), the morula rate at 144 hpi, blastocysts, and advanced blastocysts were recorded at 168 and 180 hpi (days 7 and 7.5, respectively) as percentage of cleaved zygotes. In Experiments 3 and 4, early and non-expanded blastocysts (mid blastocysts) were graded as blastocysts, while expanded, hatching, and hatched blastocysts were categorized as advanced blastocysts. Embryos were frozen at – 80 °C in phosphate-buffered saline (PBS) and polyvinyl alcohol (PVA) medium at 1.0 % (PBS-PVA) until gene expression analysis (Experiments 1, 2, and 3) and differential embryonic cell counting (Experiment 4).

Differential staining in blastocysts (blastocysts and expanded blastocysts)

Day 7.5 advanced blastocysts were differentially stained by applying a modified technique (Thouas 2001) as previously described (Gutiérrez-Añez et al. 2021) to determine the number of inner cell mass (ICM) and trophectoderm (TE) cells. Briefly, embryos were incubated in 500 µL PBS-PVA solution containing 0.75% Triton X-100 and 0.2 mg/ml propidium iodide (Solution 1) at 38°C for 30 seconds. Next, blastocysts were transferred into 500 µL of a fixative solution containing 100% ethanol with 25 µg/ml of bisbenzimide (Hoechst 33258) (Solution 2) at room temperature. Cell counting was visually determined using fluorescence microscopy (470–490 nm excitation filter) (Olympus BX60F, Tokyo, Japan) at 400fold magnifications. Serial pictures were

recorded using an attached digital camera (Olympus DP71, Tokyo, Japan). The nuclei of TE and ICM cells were differentially stained and visualized as either red/pink or blue, respectively.

Gene expression analysis

Quantification of the relative expression of the developmentally important genes Cadherin 1 (*CDH1*), caudal type homeobox 2 (*CDX2*), the regulator of MAP kinase activity (*MAPK13*), and the ion exchange and water transport genes aquaporin 3 (*AQP3*), ATPase Na+/K+ transporting subunits alpha 1 (*ATP1A1*), and beta 1 (*ATP1B1*) was performed using the primers listed in **Table 1**.

RNA Preparation

Poly(A)+ RNA was isolated using the Dynabeads® mRNA Direct Kit (Life Technologies) according to the manufacturer´s instruction with some modifications. Briefly, five to six pools of 3 morulae, four to seven pools of 3 day-7 expanded blastocysts, 4 to 6 pools of 3 day-8 expanded blastocysts, and 3 to 4 pools of 3 day-8 hatched blastocysts were thawed in 40 µl of lysis-binding buffer (100 mM Tris-HCL, at pH 8.0, 500 mM LiCl, ten (10) mM EDTA, 1% LiDS, 5.0 mM DTT). Then 3.0 pg rabbit globin mRNA was added as internal standard and incubated at room temperature for 10 minutes. Prewashed Dynabeads® Oligo d(T)25 (5 µl) were pipetted to the lysate and incubated for 15 minutes at 25 °C on a shaker for binding the poly(A)+ RNA to the beads. The beads were separated by using a Dynal MPC-E-1 magnetic separator, washed once with washing buffer A (10 mM Tris-HCL. pH 8.0, 0.15 mM LiCl, 1.0 mM EDTA, 0.1% LiDS) and two times with washing buffer B (10 mM Tris-HCL. pH 8.0, 0.15 mM LiCl, 1.0 mM EDTA). The poly(A)+ RNA was eluted from the beads by

incubation in 10 µl sterile water at 68 °C for two minutes, and the mRNA was used immediately as input for the reverse transcription reaction.

Reverse transcription (RT)

Reverse transcription was conducted in a 20 µl volume consisting of 4.0 µl of 5x RT buffer (Life Technologies), 1.0 mM dNTP (Life Technologies), 20 Units RNAsin® (Life Technologies), 100 Units M-MLV reverse transcriptase (Life Technologies, Cat.no. 28025-013) and 2.5 µM random hexamer primer (Life Technologies). Afterward, samples were incubated at 25°C for ten minutes for primer annealing and then incubated at 42°C for one hour. Finally, the samples were heated to 90°C for 5 minutes. The cDNA was diluted to a concentration of 0.1 embryo equivalent/µl. Two µl were used for Real-Time PCR amplification.

Real-time qPCR

Real-time qPCR was carried out in 96-Well Optical Reaction Plates (Life Technologies). The PCR mix in each well included 10 µl of 2x Power SYBR_Green PCR Master Mix (Life Technologies, Cat.no. A25742), and 0.1 µM of each of the forward and reverse primers and 2.0 µl of cDNA in a final reaction volume of 20 µl.

The PCR reaction was carried out in an ABI 7500 Fast Real-Time System (Applied Biosystems) using the following program: denaturation and activation of the Taq Polymerase during 10 min at 95 °C followed by 40 cycles at 95 °C during 15 seconds and 60 °C for one minute, followed by slow heating for displaying a dissociation curve of the product. Data generated by the Sequence Detection Software 1.4 were transferred to Microsoft Excel for analysis. Differential mRNA expression of each target gene was calculated by the Relative Standard Curve Method. A cDNA dilution from pooled blastocysts mRNA was included on every plate to yield standard

curves for each gene. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and rabbit globin were used as the internal controls. Standard curves were used to calculate the relative concentration of each target gene to be normalized to the geometric mean from Globin/Gapdh expression results for each sample.

Statistical Analysis

Categorical data from IVF outcomes and embryo developmental rates (e.g., cleavage, morulae, blastocysts, and advanced blastocysts) were compared using the logistic procedure (PROC LOGISTIC, SAS®). Additionally, the analysis of maximum likelihood estimates was performed to determine the odds ratio and the confidence interval limits for relative risks for embryo developmental rates on overall IVP data through the logistic procedure from SAS®.

For cell counts, total ratios were statistically analyzed through multiple comparisons analysis (Least Squares Means procedure) from the same statistical package. Either Tukey or Student's t-test multiple comparisons were applied to compare the effects of treatment and embryo category. Student's test was used to evaluate interactions between embryo stage and treatment due to the small sample size of each embryo category. All data were expressed as mean ± SEM (standard error of the mean).

Statistical analysis in the gene expression studies was performed among experimental groups using one-way analysis of variance (ANOVA), followed by Tukey test for multiple comparisons. In Experiment 3 (day 7.5: 180 hpi), expanded and hatched blastocysts were independently analyzed in each category of the embryo stage among experimental groups. All data are expressed as the mean values for each set of data ± SEM unless otherwise stated.

Significant differences were defined at $p<0.05$ in all statistical analyses.

Results

In vitro embryo production (IVP)

Logistic regression analysis revealed similar cleavage rates (p>0.05) amongst treatment groups throughout the different experiments (Experiment 1, 2, and 3) (**Table 2**). However, in Experiment 1 [production of morula stages (day 6: 144 hpi)], the percentage of morulae from the MT 1.0 nM and MT 0.01 nM were greater (p<0.05) than that found in the Control Group (53.3% and 52.7% *vs*. 41.1%, respectively). Likewise, the percentage of Total embryos was greater in the MT 0.01 nM (60.9%) compared to Control (p<0.05; 47.8%) and Sham Control group (p=0.051; 51.6%). In Experiment 2, the percentage of total embryos found in the MT 0.01 nM (55.3%) and MT 1.0 nM (52.7%) groups was greater (p<0.05) compared to the Sham control (40.2%). Likewise, the percentage of compacted morulae in the Sham control group (2.4%), differed (p<0.05) from that observed in MT 0.01 nM (13.6%), MT 1.0 nM (11.6%), and Control (8.7%). Statistical analysis did not reveal differences (p>0.05) in any of the variables evaluated in Experiment 3] (**Table 2**).

Total embryo developmental rates across all studies

As shown in **Table 3**, the logistic procedure analysis revealed that MT 0.01 nM was compatible with greater developmental rates compared to the Control group (p<0.01) and the Sham control (p<0.05) (53.8% vs. 45.9% and 46.0%, respectively). Additionally, the odds ratio (OR) for the occurrence of embryo development indicated that COCs and zygotes exposed to the lower melatonin concentration (0.01 nM) had a 1.374 times, or 37.4% [95% Wald Confidence Limits: 1.084-1.742] and 1.367 (36.7%) [95% Wald Confidence Limits: 1.080–1.729] higher chance to become a viable embryo compared to the Control and Sham control groups, respectively.

When comparing the combined results for the two control groups (Control and Sham-Control) with the melatonin groups (MT 1.0 nM and MT 0.01 nM), we found that melatonin supplementation increased the percentage of zygotes cultured that became viable embryos significantly (p<0.01) over the controls (Control groups: 45.9% vs. Melatonin groups: 52.0%) (**Table** 3). The OR for the combined effect was 1.274 [95% Wald Confidence Limits: 1.075–1.509], meaning that COCs and zygotes exposed to melatonin had a 27.4% higher chance of becoming a viable embryo compared to the control groups.

Differential embryonic cell counting

Results of the embryonic cell counting study are summarized in **Tables 4** and **5**. As shown in **Table 4**, the number of TE cells was significantly higher (p<0.05) in the MT 1.0 nM (101.6±5.2) and MT 0.01 nM (101.2±4.9) groups compared with the Control (86.5±5.1) and Sham control (83.6±5.0) groups. Likewise, the number of total embryonic cells was significantly higher (p<0.05) in the MT 1.0 nM (143.7±6.5) and MT 0.01 nM (147.3±6.1) groups, respectively, compared with the Sham control (122.5±6.2). The lower concentration of melatonin (MT 0.01 nM) marginally increased (p=0.053) the total number of cells compared to the Control group (130.3±6.3) (**Table 4**).

As expected, it was observed that hatched blastocysts had a significantly higher number of TE (p<0.01) and Total cells (p<0.01) compared to that expanded blastocysts (103.6±4.2 and 148.5±5.3 vs. 82.8±2.8 and 123.4±3.5, respectively) (**Table 4**). However, within the Sham control group both expanded and hatched blastocysts had statistically the same number of TE (p>0.05) and Total embryonic cells (p>0.05) (79.8±5.1 vs. 87.4±8.6 and 119.7±6.3 vs. 125.4±10.7, respectively) (**Table 5**).

Similarly, the number of TE cells observed in the Control group only marginally differed (p=0.06) from that observed in hatched blastocysts (76.6±5.5 vs. 96.3±8.6, respectively), while within both MT 1.0 nM and MT 0.01 nM groups, hatched blastocysts showed a significantly higher (p<0.05) number of TE and Total cells than that found in expanded blastocysts [MT 1.0 nM (114.9±8.6 vs. 88.4±6.1) MT 0.01 nM (116.0±8.1 vs. 86.5±5.5, respectively)]. Moreover, the number of TE and Total embryonic cells in expanded blastocysts from both melatonin groups was similar to that observed in hatched embryos of the Control group (**Table 5**).

Gene expression analysis

Transcript levels of genes in morulae (day 6), blastocysts (day 7), and advanced blastocysts [(day 7.5)] stages are shown in **Fig. 1**, **2** and **3**, respectively. The relative abundance of genes analyzed in compacted morulae was not different amongst treatment groups (**Fig. 1**).

In blastocysts from day 7, the lower concentration of melatonin (MT 0.01 nM) was associated with a higher relative mRNA abundance of ATPase Na+/K+ transporting subunit alpha (ATP1A1, p<0.05) and regulator of MAP kinase activity (*MAPK13*, p<0.05), whereas Cadherin 1 (*CDH1*) only tended to be higher (p=0.07) to that found in the Sham control group (**Fig. 2**).

The relative abundance of mRNA found in the higher concentration of melatonin (MT 1.0 nM) within expanded blastocysts (day 7.5, Experiment 3) was higher for aquaporin 3 (AQP3, p<0.05) and Cadherin 1 (*CDH1*, p<0.05) compared to the Control group (**Fig. 3A**). Similarly, within the hatched blastocyst stage, the relative mRNA abundance of caudal type homeobox 2 (*CDX2*) was elevated in the MT 0.01 nM (p<0.05) and Control (p<0.05) compared to the Sham control group (**Fig. 3B**). The

relative abundance of mRNA of Cadherin 1 (*CDH1*) and MAP kinase activity (*MAPK13*) found in the MT 0.01 nM tended (p=0.06) to be greater than that found in the Sham control group (**Fig. 3B**).

Discussion

Results of the present study show that melatonin added to the culture medium can improve bovine blastocyst yields in a significant manner and revealed that expression of *CDH1* and *AQP3* were significantly affected by the presence of melatonin. Our results confirm previous studies (Komninou et al. 2016; Remião et al. 2016; Zhao et al. 2015; Tian et al. 2014; Wang et al. 2014), in which an overall improvement of 5–7% of blastocyst yields was observed which could make a difference when applied in the field. We show that cumulus-oocyte complexes and embryos exposed to melatonin have a 25–30% higher likelihood of producing a viable embryo compared to untreated oocytes/embryos. In support of this assumption, melatonin increased the percentage of morulae at day six and total embryos compared to the control groups. Presumably, supplementation with melatonin leads to better early synchronized development, reflected in a higher number of good quality morulae and more good quality embryos. This assumption is further underlined by the fact that embryos derived from the melatonin treatment possessed more embryonic cells, preferably trophectodermal cells in expanded and hatched blastocysts. A higher number of embryonic cells after melatonin supplementation during IVM of viable (Tian et al. 2014; Remião et al. 2016) and inferior cumulus-oocyte complexes (Yang et al. 2017) has been reported.

The present results revealed that expanded blastocysts from day 7.5 derived from the higher melatonin concentration (MT 1.0 nM) exhibited an increased relative

abundance of mRNA for *CDH1*, one of the genes responsible for intercellular connectivity. Enhanced embryo developmental rates and an increase of cells, preferably in the trophectoderm found in the melatonin groups compared with the control groups, could be associated with the observed increased relative abundance of mRNA for *CDH1* and *AQP3*. The transcript *CDH1* is a cell surface adhesion molecule that plays a critical role in the early stages of preimplantation embryo development (Meng and Takeiki 2009). A genetic knock-out of *CDH1* in the mouse model was associated with the lack of polarized functional trophectoderm during preimplantation embryo development (Kan et al. 2007). Supplementation of culture media with Interferon-τ (*IFNT*) promoted embryo development and increased the number of embryonic cells by upregulating the expression of connexin 43 (*GJA1*) and *CDH1* (Bao et al. 2014). E-cadherin is an essential element of intercellular connection, is essential for cell adhesion and precedes the establishment of other cell-to-cell contacts, such as desmosomes and tight junctions (Watson et al. 2004).

We also found that expanded blastocysts from day 7.5, derived from the higher melatonin concentration MT 1.0 nM, exhibited an increased relative abundance of mRNA for the water transport gene Aquaporin 3 (*AQP3*) when compared to the control group. The transcript *AQP3* plays a major role in blastocyst cavitation and is involved in water movement across the trophectoderm (Barcroft et al. 2003). Significantly more blastocysts have previously been observed on day seven of embryo culture in the presence of melatonin, indicating that the hormone stimulates the development of *in vitro* produced bovine embryos (Wang et al. 2014).

The presence of melatonin during *in vitro*-produced bovine embryo culture increased expression of *CDH1* after vitrification/thawing (Wang et al. 2014), while a

down-regulation of *AQP3* was observed. In our study, the embryos had not been frozen/thawed, which might explain why the mRNA expression profiles of *AQP3* were different between the present study and the previous one from Wang et al. (2014). A lower amount of *AQP3* transcripts was found in vitrified-warmed embryos compared with their fresh counterparts (Camargo et al. 2011).

Increased *AQP3* expression in day-7.5-expanded blastocysts observed in the present study could have an important implication for applying embryo cryopreservation procedures. One of the main concerns during cryopreservation is that cells undergo extensive osmotic stress during dehydration, followed by rehydration caused by permeating cryoprotectants and ultimately become damaged by toxicity (Camargo et al. 2011). The toxicity of cryoprotectants such as glycerol or ethylene glycol is mainly dependent on its permeability to the cell membrane (Camargo et al. 2011). Thus water and solutes movement across the cell membrane via aquaporin channels is crucial role for cell viability. Important properties of aquaporin isoforms such as *AQP3* include permeability to water and glycerol homologs, also referred to as aquaglyceroporins (Kruse et al. 2006), which can enhance cell permeability and may participate in the diffusion rate of cryoprotectants during cryopreservation.

We also observed that 7.5 days hatched blastocysts derived from the lower concentration of melatonin (MT 0.01 nM) tended to have increased transcript levels for *MAPK13,* which is critical for undisturbed early development (Natale et al. 2004). MAP kinase signaling is involved in critical cellular processes such as proliferation, differentiation, development, apoptosis, stress, and inflammatory responses (Aroor and Shukla 2004). Early bovine embryos critically depend on both *MAPK* signaling and the extracellular signal-regulated kinase (*ERK*) pathway to complete development to the

blastocyst stage (Madan et al. 2005). Negrón-Pérez et al. (2018) found that *MAPK* signaling was crucial for ICM differentiation via restricting epiblast cell numbers, which in turn is critical for the second differentiation step between days 7 and 9 of bovine embryo development (Gordon 2003).

The improved embryo quality observed after melatonin exposure is thought to be mainly due to its antioxidant properties (Remião et al. 2016). However, the present data suggest that genes encoding for *CDH1*, *MAPK13*, and *AQP3* could also be linked in the improved embryo quality competence after melatonin treatment. A recent report showed that the melatonin effects on fertility of heat-stressed cows might involve other functions rather than antioxidant properties (Ortega et al. 2016).

Interestingly, results from our sham control group (i.e., vehicle ethanol added to medium) suggest an impaired embryo cell counting at the hatched blastocyst stage. Our data suggest that melatonin could protect embryos against ethanol mediated impairment. In the current experiment, melatonin was dissolved in ethanol, primarily due to its low solubility in water (Zhang et al. 2020). The potentially deteriorating effects of ethanol on embryo development should be considered when planning experiments in which the candidate molecule has to be dissolved in a suitable carrier. A previous study that evaluated the effects of ethanol and dimethylsulphoxide (DMSO) on nuclear and cytoplasmic maturation of bovine cumulus-oocyte complexes concluded that even small doses of both dissolving solutions could cause profound adverse effects on bovine *in vitro* maturation and embryo development (Avery and Greve 2000). A low DMSO concentration (0.1%–0.5%) had positive effects on early embryo development indicated by better quality embryos and increased maturation and blastocyst rates (Stöhr et al., 2015; Ynsaurralde-Rivolta et al. 2020). An alternative could be to use alternative

vehicles such as or propylene glycol (Cheung et al. 2006). Very recently, a new melatonin sulfonate derivative sodium 4-(3-(2-acetamidoethyl)-5-methoxy-1H-indol-1-yl) butane-1-sulfonate (MLTBS) showed ~ 700 times higher water solubility and lower cytotoxicity than natural melatonin (Zhang et al. 2020). Further studies to address these issues are justified.

In conclusion, results of the present study revealed improved bovine *in vitro* embryo developmental rates by supplementing the medium with melatonin. Our results indicate that the developmentally-related gene type 1 epithelial cadherin 1 (*CDH1*) and the ion exchange and water transport gene aquaporin 3 (*AQP3*) are critically involved in the enhanced bovine cumulus-oocyte competence and embryo development *in vitro after melatonin treatment*. We also report for the first time that the use of ethanol could eventually impair embryo development, as shown by alterations of embryonic cell counting and the expression of *ATP1A1*, *CDH1*, *CDX2*, and *MAPK13*. Less harmful vehicles for dissolving melatonin should be explored.

Conflicts of interest

No conflicts of interest occurred to carry out this experiment.

Author contributions

Juan Carlos Gutiérrez-Añez (JCGA), Andrea Lucas-Hanh (ALH) and Heiner Niemann (HN) conceived and designed the experiments. *In vitro* fertilization experiments were performed by JCGA and Patrick Aldag. Doris Herrmann performed the gene expression analysis. JCGA made the acquisition, analysis and interpretation of data and wrote the original manuscript. HN and ALH provided the resources and

contributed to the interpretation of data. All authors reviewed the article. HN made the supervision, validation, writing and editing, and the final version's approval.

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References

Aroor, A.R., and Shukla, S.D. (2004). MAP kinase signaling in diverse effects of ethanol. Life Sci. **74**(19), 2339-64. doi.org/10.1016/j.lfs.2003.11.001

Avery, B., Greve, T. (2000). Effects of ethanol and dimethylsulphoxide on nuclear and cytoplasmic maturation of bovine cumulus-oocyte complexes. Mol Reprod Dev, **55**(4), 438-445. doi.org/10.1002/(sici)1098-2795(200004)55:4%3C438::aid-mrd12%3E3.0.co;2-y

Bao, Z.J., Zhao, S., Haq, I.U., Zeng, S.M. (2014). Recombinant bovine interferon-τ enhances in vitro development of bovine embryos by upregulating expression of connexin 43 and E-cadherin. J Dairy Sci. **97**(11), 6917-6925. doi.org/10.3168/jds.2014-8106

Barcroft, L.C., Offenberg, H., Thomsen, P., Watson, AJ. (2003). Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation. Dev Biol. **256**(2), 342-354. doi.org/10.1016/s0012-1606(02)00127-6

Braude, P., Bolton, V., Moore, S. (1988). Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature, **332**(6163), 459-461. doi.org/10.1038/332459a0

Camargo, L.S., Boite, M.C., Wohlres-Viana, S., Motam G.B., Serapiaom R.V., Sam W.F., Vianam J.H., Nogueira, L.A. (2011). Osmotic challenge and expression of aquaporin 3 and Na/K ATPase genes in bovine embryos produced in vitro. Cryobiology. **63**(3), 256-262. doi.org/10.1016/j.cryobiol.2011.09.135

Cheung, R.T., Tipoe, G.L., Tam, S., Ma, E.S., Zou, L.Y., Chan, P.S. (2006). Preclinical evaluation of pharmacokinetics and safety of melatonin in propylene glycol for intravenous administration. J Pineal Res. **41**(4), 337-343. doi.org/10.1111/j.1600-079x.2006.00372.x

Goissis, M.D., and Cibelli, J.B. (2014). Functional characterization of CDX2 during bovine preimplantation development in vitro. Mol Reprod Dev. **81**(10), 962-970. doi.org/10.1002/mrd.22415

Gordon, I. R. (2003). Laboratory production of cattle embryos, Biotechnology in agriculture series, N° 27, (2nd ed). Pp. 220-270. (CAB International, Wallingford, U.K)

Gutiérrez-Añez, J.C., Lucas-Hahn, A., Hadeler, K.G., Aldag, P., Niemann, H. (2021). Melatonin enhances in vitro developmental competence of cumulus-oocyte

complexes collected by ovum pick-up in prepubertal and adult dairy cattle. Theriogenology. **161**, 285-293. doi.org/10.1016/j.theriogenology.2020.12.011

Houghton, F.D., Humpherson, P.G., Hawkhead, J.A., Hall, C.J., Leese, H.J. (2003). Na+, K+, ATPase activity in the human and bovine preimplantation embryo. Dev Biol. **263**(2), 360-366. doi.org/10.1016/j.ydbio.2003.07.014

Johnson, M.H., and McConnell, J.M. (2004). Lineage allocation and cell polarity during mouse embryogenesis. Semin Cell Dev Biol. **15**(5), 583-597. doi.org/10.1016/j.semcdb.2004.04.002

Kan, N.G., Stemmler, M.P., Junghans, D., Kanzler, B., de Vries, W.N., Dominis, M., Kemler, R. (2007). Gene replacement reveals a specific role for E-cadherin in the formation of a functional trophectoderm. Development. **134**(1), 31-41. doi.org/10.1242/dev.02722

Kim, E.H., Kim, G.A., Taweechaipaisankul, A., Lee, S.H., Qasim, M., Ahn, C., Lee, B.C. (2019). Melatonin enhances porcine embryo development via the Nrf2/ARE signaling pathway. J Mol Endocrinol. **63**(3), 175-185. doi.org/10.1530/jme-19-0093

Komninou, E.R., Remião, M.H., Lucas, C.G., Domingues, W.B., Basso, A.C., Jornada, D.S., Deschamps, J.C., Beck, R.C., Pohlmann, A.R., Bordignon, V., Seixas, F.K., Campos, V.F., Guterres, S.S., Collares, T. (2016). Effects of Two Types of Melatonin-Loaded Nanocapsules with Distinct Supramolecular Structures: Polymeric (NC) and Lipid-Core Nanocapsules (LNC) on Bovine Embryo Culture Model. PLoS One. **11**(6):e0157561. doi.org/10.1371/journal.pone.0157561

Kruse, E., Uehlein, N., Kaldenhoff, R. (2006). The aquaporins. Genome Biol. **7**(2):206

Loren, P., Sánchez, R., Arias, M.E., Felmer, R., Risopatrón, J., Cheuquemán, C. (2017). Melatonin Scavenger Properties against Oxidative and Nitrosative Stress: Impact on Gamete Handling and In Vitro Embryo Production in Humans and Other Mammals. Int J Mol Sci. **18**(6):1119. doi.org/10.3390/ijms18061119

MacPhee, D.J., Jones, D.H., Barr, K.J., Betts, D.H., Watson, A.J., Kidder, G.M. (2000). Differential involvement of Na(+),K(+)-ATPase isozymes in preimplantation development of the mouse. Dev Biol. **222**(2), 486-498. doi.org/10.1006/dbio.2000.9708

Madan, P., Calder, M.D., Watson, A.J. (2005). Mitogen-activated protein kinase (MAPK) blockade of bovine preimplantation embryogenesis requires inhibition of both p38 and extracellular signal-regulated kinase (ERK) pathways. Reproduction. **130**(1), 41-51. doi.org/10.1530/rep.1.00554

Maekawa, M., Yamamoto, T., Tanoue, T., Yuasa, Y., Chisaka, O., Nishida, E. (2005). Requirement of the MAP kinase signaling pathways for mouse preimplantation development. Development. **132**(8), 1773-1783. doi.org/10.1242/dev.01729

Maître, J.L., Niwayama, R., Turlier, H., Nédélec, F., Hiiragi, T. (2015). Pulsatile cell-autonomous contractility drives compaction in the mouse embryo. Nat Cell Biol. **17**(7), 849-855. doi.org/10.1038/ncb3185

Meng, W., and Takeichi, M. (2009). Adherens junction: molecular architecture and regulation. Cold Spring Harb Perspect Biol. **1**(6):a002899. doi.org/10.1101/cshperspect.a002899

Nagina, G., Asima, A., Nemat, U., Shamim, A. (2016). Effect of melatonin on maturation capacity and fertilization of Nili-Ravi buffalo (Bubalus bubalis) oocytes. Open Vet J. **6**(2), 128-134. doi.org/10.4314/ovj.v6i2.9

Natale, D.R., Paliga, A.J., Beier, F., D'Souza, S.J., Watson, A.J. (2004). p38 MAPK signaling during murine preimplantation development. Dev Biol. **268**(1), 76-88. doi.org/10.1016/j.ydbio.2003.12.011

Negrón-Pérez, and V.M., Hansen, P.J. (2018). Role of yes-associated protein 1, angiomotin, and mitogen-activated kinase kinase 1/2 in development of the bovine blastocyst. Biol Reprod. **98**(2), 170-183. doi.org/10.1093/biolre/iox172

Ortega, M.S., Rocha-Frigoni, N.A.S., Mingoti, G.Z., Roth, Z., Hansen, P.J. (2016). Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in HSPA1L. J Dairy Sci. **99**(11), 9152-9164. doi.org/10.3168/jds.2016-11501

Papis, K., Poleszczuk, O., Wenta-Muchalska, E., Modlinski, J.A. (2007). Melatonin effect on bovine embryo development in vitro in relation to oxygen concentration. J Pineal Res. **43**(4), 321-326. doi.org/10.1111/j.1600-079x.2007.00479.x

Remião, M.H., Lucas, C.G., Domingues, W.B., Silveira, T., Barther, N.N., Komninou, E.R., Basso, A.C., Jornada, D.S., Beck, R.C., Pohlmann, A.R., Junior, A.S., Seixas, F.K., Campos, V.F., Guterres, S.S., Collares, T. (2016). Melatonin delivery by nanocapsules during in vitro bovine oocyte maturation decreased the reactive oxygen species of oocytes and embryos. Reprod Toxicol. **63**: 70-81. doi.org/10.1016/j.reprotox.2016.05.016

Sakurai, N., Takahashi, K., Emura, N., Fujii, T., Hirayama, H., Kageyama, S., Hashizume, T., Sawai, K. (2016). The Necessity of OCT-4 and CDX2 for Early

Development and Gene Expression Involved in Differentiation of Inner Cell Mass and Trophectoderm Lineages in Bovine Embryos. Cell Reprogram. **18**(5), 309-318. doi.org/10.1089/cell.2015.0081

Schultz, R.M. (1993). Regulation of zygotic gene activation in the mouse. Bioessays. **15**(8), 531-538. doi.org/10.1002/bies.950150806

Soto-Heras, S., Catalá, M.G., Roura, M., Menéndez-Blanco, I., Piras, A.R., Izquierdo, D., Paramio, M.T. (2019). Effects of melatonin on oocyte developmental competence and the role of melatonin receptor 1 in juvenile goats. Reprod Domest Anim. **54**(2), 381-390. doi.org/10.1111/rda.13378

Soto-Heras, S., Roura, M., Catalá, M.G., Menéndez-Blanco, I., Izquierdo, D., Fouladi-Nashta, A.A., Paramio, M.T. (218). Beneficial effects of melatonin on in vitro embryo production from juvenile goat oocytes. Reprod Fertil Dev. **30**(2), 253-261. doi.org/10.1071/rd17170

Sponchiado, M., Gomes, N.S., Fontes, P.K., Martins, T., Del Collado, M., Pastore, A.A., Pugliesi, G., Nogueira M.F.G., Binelli, M. (2017). Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PLoS One. **12**(4):e0175954. doi.org/10.1371/journal.pone.0175954

Stöhr, J., Grothmann, H., Wrenzycki, C. (2015). 55 EFFECT OF DIMETHYL SULFOXIDE SUPPLEMENTATION ON BOVINE IN VITRO EMBRYO DEVELOPMENT. Reprod. Fertil. Dev. **28**(1-2), 157-158

Telford, N.A., Watson, A.J., Schultz, G.A. (1990). Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev. **26**(1), 90-100. doi.org/10.1002/mrd.1080260113

Thouas, G.A., Korfiatis, N.A., French, A.J., Jones, G.M., Trounson, A.O. (2001). Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod Biomed Online. **3**(1), 25-29. doi.org/10.1016/s1472-6483(10)61960-8

Tian, X., Wang, F., He, C., Zhang, L., Tan, D., Reiter, R.J., Xu, J., Ji, P., Liu, G. (2014). Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. J Pineal Res. **57**(3), 239-247. doi.org/10.1111/jpi.12163

Tian, X., Wang, F., Zhang, L., He, C., Ji, P., Wang, J., Zhang, Z., Lv, D., Abulizi, W., Wang, X., Lian, Z., Liu, G. (2017). Beneficial Effects of Melatonin on the In Vitro Maturation of Sheep Oocytes and Its Relation to Melatonin Receptors. Int J Mol Sci. **18**(4):834. doi.org/10.3390/ijms18040834

Violette, M.I., Madan, P., Watson, A.J. (2006). Na+/K+ -ATPase regulates tight junction formation and function during mouse preimplantation development. Dev Biol. **289**(2), 406-419. pubmed.ncbi.nlm.nih.gov/16356488/

Wang, F., Tian, X., Zhou, Y., Tan, D., Zhu, S., Dai, Y., Liu, G. (2014). Melatonin improves the quality of in vitro produced (IVP) bovine embryos: implications for blastocyst development, cryotolerance, and modifications of relevant gene expression. PLoS One. **9**(4):e93641. doi.org/10.1371/journal.pone.0093641

Wang, T., Gao, Y.Y., Chen, L., Nie, Z.W., Cheng, W., Liu, X., Schatten, H., Zhang, X., Miao, Y.L. (2017). Melatonin prevents postovulatory oocyte aging and promotes subsequent embryonic development in the pig. Aging (Albany NY). **9**(6), 1552-1564. doi.org/10.18632/aging.101252

Watson, A.J., Natale, D.R., Barcroft, L.C. (2004). Molecular regulation of blastocyst formation. Anim Reprod Sci. **82-83**, 583-592. doi.org/10.1016/j.anireprosci.2004.04.004

Yang, L., Wang, Q., Cui, M., Li, Q., Mu, S., Zhao, Z. (2020). Effect of Melatonin on the In Vitro Maturation of Porcine Oocytes, Development of Parthenogenetically Activated Embryos, and Expression of Genes Related to the Oocyte Developmental Capability. Animals (Basel). **10**(2):209. doi.org/10.3390/ani10020209

Yang, M., Tao, J., Chai, M., Wu, H., Wang, J., Li, G., He, C., Xie, L., Ji, P., Dai, Y., Yang, L., Liu, G. (2017). Melatonin Improves the Quality of Inferior Bovine Oocytes and Promoted Their Subsequent IVF Embryo Development: Mechanisms and Results. Molecules. **22**(12):2059. doi.org/10.3390/molecules22122059

Ynsaurralde-Rivolta, A.E., Suvá, M., Luchetti, C.G., Bevacqua, R.J., Munilla, S., Rodriguez-Alvarez, L., Velasquez, A., Briski, O., Lombardo, D., Salamone, D. (2020). DMSO supplementation during in vitro maturation of bovine oocytes improves blastocyst rate and quality. Theriogenology. **148**, 140-148. doi.org/10.1016/j.theriogenology.2020.02.045

Zhang, J., Yan, X., Tian, Y., Li, W., Wang, H., Li, Q., Li, Y., Li, Z., Wu, T. (2020). Synthesis of a New Water-Soluble Melatonin Derivative with Low Toxicity and a Strong Effect on Sleep Aid. ACS Omega. **5**(12), 6494-6499. doi.org/10.1021/acsomega.9b04120

Zhao, X.M., Min, J.T., Du, W.H., Hao, H.S., Liu, Y., Qin, T., Wang, D., Zhu, H.B. (2015). Melatonin enhances the in vitro maturation and developmental potential of bovine oocytes denuded of the cumulus oophorus. Zygote. **23**(4), 525-536. doi.org/10.1017/s0967199414000161

Zhao, X.M., Wang, N., Hao, H.S., Li, C.Y., Zhao, Y.H., Yan, C.L., Wang, H.Y., Du, W.H., Wang, D., Liu, Y., Pang, Y.W., Zhu, H.B. (2018). Melatonin improves

the fertilization capacity and developmental ability of bovine oocytes by regulating cytoplasmic maturation events. J Pineal Res. **64**(1). doi.org/10.1111/jpi.12445

Zou, H., Chen, B., Ding, D., Gao, M., Chen, D., Liu, Y., Hao, Y., Zou, W., Ji, D., Zhou, P., Wei, Z., Cao, Y., Zhang, Z. (2020). Melatonin promotes the development of immature oocytes from the COH cycle into healthy offspring by protecting mitochondrial function. J Pineal Res. **68**(1):e12621. doi.org/10.1111/jpi.12621

Table 1. Primers sequences used for the real-time qPCR

Gene	Primer sequences: (5´-3´)	Fragment size/bp	Accession no.
AQP3	F: CTGGGCGCTGGAATTATCTTC R: GCCCGAAACAATAAGCTGGTT	78	NM_001079794
ATP1A1	F: GAACTTCATCGCAAATATGGAACA R: GGGTCCATCTCTGGCTAGGAT	84	NM_001076798
ATP1B1	F: GAAGGAGTTTTTGGGCAGGAC R: AGCATCACTTGGATGGTTCCG	111	NM_001035334.1
CDH1	F: GACACCCGGGACAATGTGTA R: GCCCCTATGTAACTGGCTCAA	86	NM_001002763
CDX2	F: CAGAGAGGCAGGTTAAAATTTGGT R: CTGCTGTTGCAACTTCTTCTTGTT	80	NM_001206299.1
MAPK13	F: GAAAACGTCATCGGGCTTCTG R: AGCTGAGTGGATGTACTTAAGACCC	189	NM_001014947.1
GAPDH	CCCACTCCCAACGTGTCTGT CCTGCTTCACCACCTTCTTGAT	89	NM_001034034.2
Rabbit Globin	F: TACTTCCCCCACTTCGACTTCA R: AGGGCTTCGGACACCTTCTT	74	NM_001082389.2

F: forward; R: reverse

Table 2. Embryo developmental rates of cumulus-oocyte complexes and zygotes exposed to melatonin during *in vitro* culture (COCs from 22 IVF cycles)

Experiment 1 [Morulae stage (day 6); six IVF cycles]									
	Zygotes	**Cleavage**		**Morulae**		**Blastocyst**		**Total**	
Treatment	*n*	*n*	%	*n*	%	*n*	%	*n*	%
Control	254	207	81.5	85	41.1[b]	14	6.8	99	47.8[b]
Sham control	272	215	79.0	97	45.1[ab]	14	6.5	111	51.6[ab#]
MT 1.0 nM	211	165	78.2	88	53.3[a]	9	5.5	97	58.8[a]
MT 0.01 nM	279	220	78.9	116	52.7[a]	18	8.2	134	60.9[a#]
Average	**1016**	**807**	**79.4**	**386**	**47.8**	**55**	**6.8**	**441**	**54.6**
Experiment 2 [Blastocyst stage (day 7); six IVF cycles]									
	Zygotes	**Cleavage**		**Morulae**		**Blastocyst**		**Total**	
Control	158	127	80.4	11	8.7[a]	49	38.6	60	47.2[ab]
Sham control	164	127	77.4	3	2.4[b]	48	37.8	51	40.2[b]
MT 1.0 nM	157	129	82.2	15	11.6[a]	53	41.1	68	52.7[a]
MT 0.01 nM	167	132	79.0	18	13.6[a]	55	41.7	73	55.3[a]
Average	**646**	**515**	**79.7**	**47**	**9.1**	**205**	**39.8**	**252**	**48.9**
Experiment 3 [Advanced blastocyst stage (day 7.5); ten IVF cycles]									
	Zygotes	**Cleavage**		**Early Bl.**		**Advanced Bl.**		**Total**	
Control	247	198	80.2	35	17.7	50	25.3	85	42.9
Sham control	249	208	79.4	37	17.8	54	26.0	91	43.8
MT 1.0 nM	247	203	77.8	31	15.3	52	25.6	83	40.9
MT 0.01 nM	253	215	84.9	48	22.3	50	23.3	98	45.6
Average	**996**	**824**	**82.7**	**151**	**18.3**	**206**	**25.0**	**357**	**43.3**

[a,b] Logistic procedure (Chi-square): Percentages among columns with different superscripts differ ($p<0.05$). [#] ($p=0.051$). Control: without any supplements. Sham control: Ethanol in the IVP medium. MT 1.0 and MT 0.01 nM: Melatonin in the IVP medium. Experiment 1: day 6. Experiment 2: day 7. Experiment 3: day 7.5: Early Bl.: early blastocyst (Early + mid blastocysts); Advanced Bl.: advanced blastocysts (expanded + hatched blastocysts).

Table 3. Overall embryo developmental rates of cumulus-oocyte complexes and zygotes exposed to melatonin during *in vitro* culture (COCs from 22 IVF cycles)

	Zygotes	**Cleavage**		**Suitable embryos**	
Treatment	*n*	*n*	%	*n*	%
Control	659	532	80.7	244	45.9[b]
Sham control	685	550	80.3	253	46.0[b]
MT 1.0 nM	615	497	80.8	248	49.9[ab]
MT 0.01 nM	699	567	81.1	305	53.8[a]
Average	**2658**	**2146**	**80.7**	**1050**	**48.9**
Logistic procedure: Individual treatment effect (Control *vs* MT 0.01 nM)					
		Cleavage		**Suitable embryos**	
	p value	0.858		0.009	
	Odds ratio	1.025		1.37	
	95% Wald CL	0.782–1.344		1.084–1.742	
	Zygotes	**Cleavage**		**Suitable embryo**	
	n	*n*	%	*n*	%
Control groups	1344	1082	80.5	497	45.9[b]
Melatonin (MT) groups	1314	1064	80.9	553	52.0[a]
Average	**2658**	**2146**	**80.7**	**1050**	**48.9**
Logistic procedure: Combined treatment effect (Control *vs* Melatonin)					
		Cleavage		**Suitable embryos**	
	p value	0.7596		0.005	
	Odds ratio	1.031		1.274	
	95% Wald CL	0.850–1250		1.075–1.509	

[a,b] Logistic procedure (Chi-square): Percentages among columns with different superscripts differ ($p<0.05$). 95% Wald CL: 95% Wald Confidence Limits for relative risks. Control: without any supplements. Sham control: Ethanol in the IVP medium. MT 1.0 and MT 0.01 nM: Melatonin in the IVP medium. An odds ratio of 1.274 indicates a 27.4% increase in the odds of achieved any value of suitable embryo developmental rates (day 6: compacted morulae + blastocysts; day 7: blastocysts; day 7.5: advanced blastocysts (expanded + hatched blastocysts) when cumulus-oocyte complexes and zygotes were exposed to melatonin compared to control.

Table 4. Effects of melatonin supplementation during *in vitro* embryo production on embryonic ICM and TE cell counts in blastocysts from slaughterhouse-derived ovaries (Embryos from five IVF cycles)

	Treatment and embryo stage effect							
	Treatment effect					**Embryo stage effect**		
Variable	**Control**	**Sham**	**MT 1.0 nM**	**MT 0.01 nM**	***p value***	**Expanded**	**Hatched**	***P value***
TE	86.5±5.1^{b}	83.6±5.0^{b}	101.6±5.2^{a}	101.2±4.9^{a}	0.02	82.8±2.8^{b}	103.6±4.2^{a}	0.00009
ICM	43.8±2.9	39.0±2.8	42.0±3.0	46.1±2.8	0.3	40.5±1.6	44.9±2.4	0.1
Total	130.3±6.3$^{ab\#}$	122.5±6.2^{b}	143.7±6.5^{a}	147.3±6.1$^{a\#}$	0.02	123.4±3.5^{b}	148.5±5.3^{a}	0.0001
TE:Total ratio	0.66±0.017	0.67±0.016	0.71±0.017	0.68±0.016	0.3	0.67±0.009	0.69±0.014	0.2
ICM:Total ratio	0.33±0.017	0.32±0.016	0.292±0.017	0.31±0.016	0.3	0.33±0.009	0.30±0.014	0.2

Least Square Means Procedure: Values are the means ± SEM. [a,b] Treatment effect [Control (*n*: 24); Sham control (*n*: 27), MT 1.0 nM (*n*: 21); MT 0.01 nM (*n*: 25)]; means within rows amongst treatments with different superscripts differ ($p<0.05$; All Pairwise Comparisons). Embryo stage effect [Expanded (*n*: 68); Hatched (*n*: 29)] means within rows amongst embryo categories with different superscripts differ ($p< 0.05$). [#] refers to marginal significance within comparisons [Treatment effect: Control vs. MT 0.01 nM (Total cells: $p=0.05$).

Table 5. Effects of melatonin supplementation during *in vitro* embryo production and embryo stage (expanded vs. hatched blastocysts) on embryonic ICM and TE cell counts in blastocysts from slaughterhouse-derived ovaries (Embryos from five IVF cycles)

	Treatment by embryo stage effect							
	Control		**Sham**		**MT 1.0 nM**		**MT 0.01 nM**	
Variable	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**
TE	76.6±5.5$^{b\#}$	96.3±8.6$^{ab\#}$	79.8±5.1^{b}	87.4±8.6^{b}	88.4±6.1^{b}	114.9±8.6^{a}	86.5±5.5^{b}	116.0±8.1^{a}
ICM	39.2±3.1	48.4±4.9	39.9±2.9	38.0±4.9	37.5±3.5	46.6±4.9	45.6±3.1	46.6±4.6
Total	115.8±6.9^{c}	144.7±10.7^{ab}	119.7±6.3^{c}	125.4±10.7^{bc}	125.9±7.6^{bc}	161.4±10.7^{a}	132.1±6.9^{bc}	162.6±10.0^{a}
TE:Total	0.66±0.02	0.67±0.03	0.66±0.02	0.69±0.03	0.71±0.02	0.71±0.03	0.65±0.02	0.71±0.03
ICM:Total	0.34±0.02	0.33±0.03	0.34±0.02	0.31±0.03	0.29±0.02	0.29±0.03	0.34±0.02	0.29±0.03

Least Square Means Procedure: Values are the means ± SEM. ^{a,b,c} Interaction effect (treatment by embryo stage effect) [Expanded: Control (*n*: 17); Sham control (*n*: 20), MT 1.0 nM (*n*: 14); MT 0.01 nM (*n*: 17); Hatched: Control (*n*: 7); Sham control (*n*: 7), MT 1.0 nM (*n*: 7); MT 0.01 nM (*n*: 8)]. ^{a,b,c} means within rows with different letters differ significantly ($p<0.05$; Student's t All Pairwise Comparisons). $^{\#}$ refers to marginal significance within comparisons [Control group: Expanded vs. Hatched (TE: $p=0.06$).

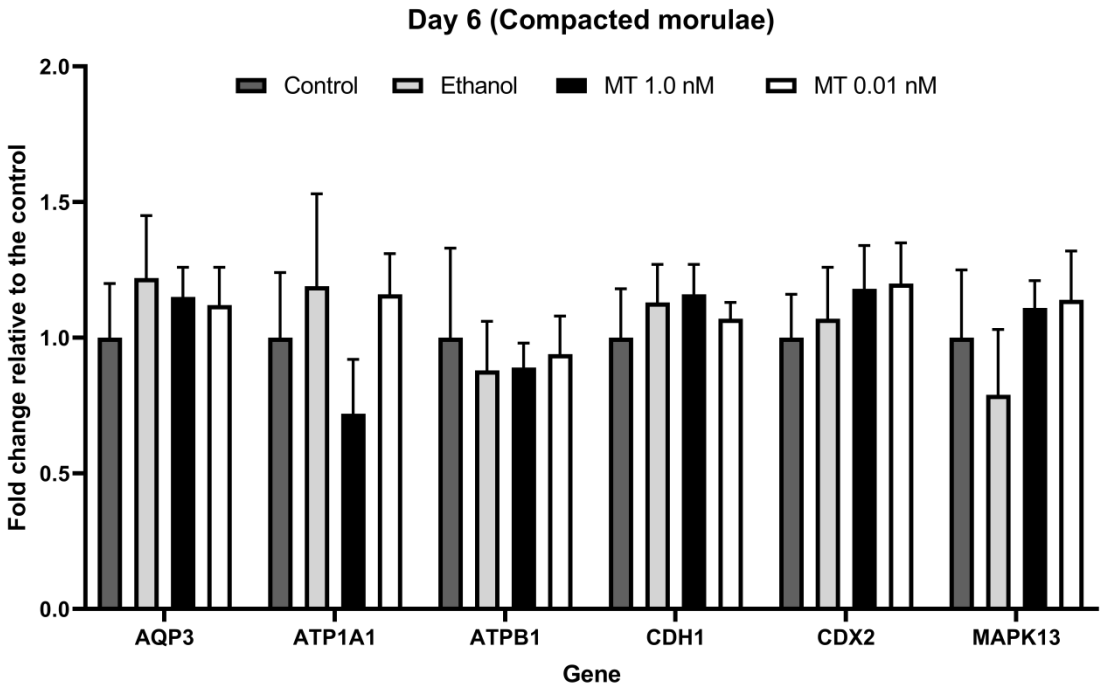

Fig. 1. Effects of melatonin on the relative mRNA abundance of 1) Developmentally-related genes such as the type 1 epithelial cadherin 1 (*CDH1*), the caudal type homeobox 2 (*CDX2*), and the regulator of MAP kinase activity (*MAPK13*), and 2) Ion exchange and water transport genes such as aquaporin 3 (*AQP3*), the ATPase Na+/K+ transporting subunits alpha 1 (*ATP1A1*), and beta 1 (*ATP1B1*) at morulae stage (Experiment 1, Day 6: 144 hpi). Data are represented as fold change relative to the control after normalizing the geometric mean from the housekeeping genes (Globin/Gapdh) and are the means ± SEM. No statistical significance (p>0.05).

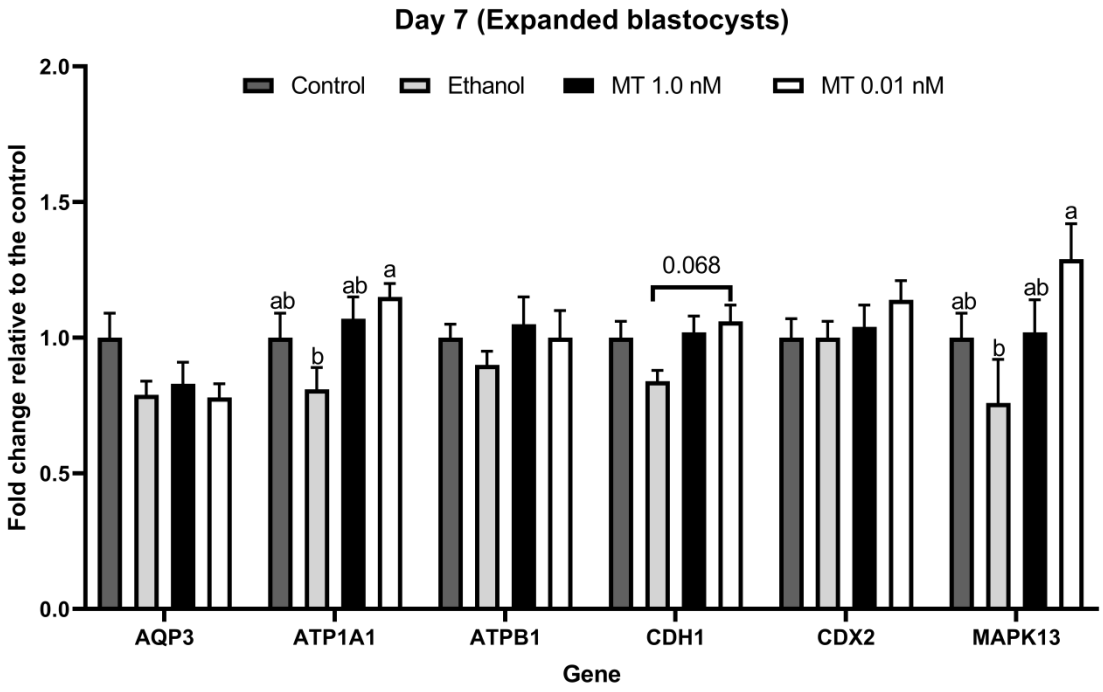

Fig. 2. Effects of melatonin on the relative mRNA abundance of 1) Developmentally-related genes such as the type 1 epithelial cadherin 1 (*CDH1*), the caudal type homeobox 2 (*CDX2*), and the regulator of MAP kinase activity (*MAPK13*), and 2) Ion exchange and water transport genes such as aquaporin 3 (*AQP3*), the ATPase Na+/K+ transporting subunits alpha 1 (*ATP1A1*), and beta 1 (*ATP1B1*) at blastocyst stage (Experiment 2, Day 7: 168 hpi). Data are represented as fold change relative to the control after normalizing the geometric mean from the housekeeping genes (Globin/Gapdh) and are the means ± SEM. Different letters (a-b) among genes between treatment groups indicates differences (p<0.05). Percentages with enclosed bracket marks among categories between treatment groups show the p values where statistical trends were found.

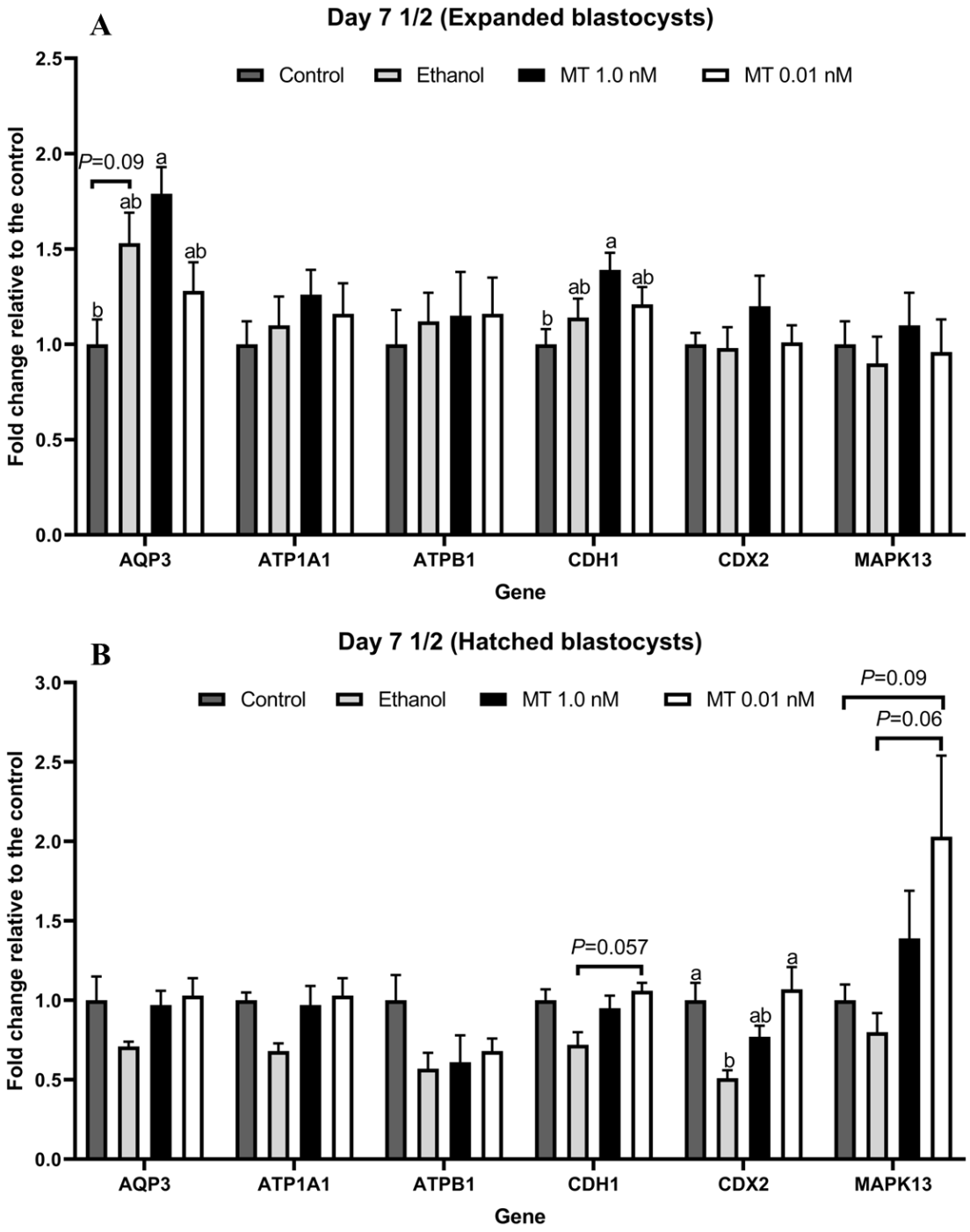

Fig. 3. Effects of melatonin on the relative mRNA abundance of 1) Developmentally-related genes such as the type 1 epithelial cadherin 1 (*CDH1*), the caudal type homeobox 2 (*CDX2*), and the regulator of MAP kinase activity (*MAPK13*), and 2) Ion exchange and water transport genes such as aquaporin 3 (*AQP3*), the ATPase Na+/K+ transporting subunits alpha 1 (*ATP1A1*), and beta 1 (*ATP1B1*) at advanced blastocyst

stage (Experiment 3, Day 7 ½: 180 hpi). A) Expanded blastocysts. B) Hatched blastocysts. Data are represented as fold change relative to the control after normalizing the geometric mean from the housekeeping genes (Globin/Gapdh) and are the means ± SEM. Different letters (a-b) among genes between treatment groups indicate differences ($p<0.05$). Percentages with bracket among categories between treatment groups show the p values where statistical trends were found.

Chapter V

5 General discussion

The goal of this dissertation was to evaluate the influence of melatonin on the developmental competence of bovine female and male gametes and preimplantation embryo development *in vitro* after IVF using oocytes from adult and prepubertal dairy cattle. We explored the role of the hormone by incorporating melatonin in different steps of *in vitro* embryo production, i.e., *in vitro* maturation, fertilization, and culture, and evaluated the effects on sperm functionality and early embryo development. Additionally, we have studied the effects of melatonin on *in vivo* harvested COCs collected via ultrasound-guided follicular aspiration (OPU) or postmortem follicular aspiration from slaughter-derived-ovaries.

5.1 Effects of melatonin on sperm functionality and subsequent fertilization ability *in vitro*: Implications for embryo development

The present results clearly show that regular fertilization of bovine cumulus-oocyte complexes was increased after IVF with post-thaw melatonin-treated sperm, concomitantly with a marked reduction of polyspermy when the medium had been supplemented with melatonin. Moreover, the reduction of polyspermy and the increase of regular mono-spermic fertilization were accompanied by an enhanced embryo development. These findings may improve IVP efficiency since fertilization failures regularly detract from the success of IVF cycles (Zhang et al., 2019).

The majority of published research supports the hypothesis that melatonin is critically involved in sperm mitochondrial physiology. This assumption is based on the fact that sperm motility mainly depends on the flagellar movement of the sperm tail,

which in turn relies on the ATP contents produced by the mitochondria located in the sperm mid-piece (Srivastava et al., 2016). Mitochondrial ATP production is an indispensable requirement for sustaining sperm-triggered Ca^{2+} oscillations during fertilization and essential to support early embryo development (Harvey, 2019). Improved progressive sperm motility has been associated with intact mitochondrial membrane potential and consequently mitochondrial functionality (Moscatelli et al., 2017). However, no significant mitochondrial membrane potential changes were observed in the present study. Further mitochondrial activity tests, such as mitochondrial volume measurements, analysis of mitochondrial permeability transition pores, and measurements of ATP contents, could provide an in-depth insight into the role of melatonin in sperm mitochondrial functionality and its effects on fertilization and early *in vitro* embryo development.

The effects of melatonin on mitochondrial function have been investigated in spermatozoa from various mammalian species [Fang et al., 2020a, 2020b (Ram); Zhang et al., 2019 (Human) Perumal et al., 2018 (Mithun)]. A recent study reported a higher mitochondrial respiratory capacity of sperm cells by increasing ATP production via the oxidative phosphorylation pathway after melatonin treatment (Fang et al., 2020b). Melatonin was found to inhibit the mitochondrial permeability transition pores and thereby prevented the release of pro-apoptotic factors into the cytoplasm, which in turn protected mitochondria from cryoinjury, promoted ATP synthesis, and improved sperm motility and viability of frozen-thawed sperm (Fang et al., 2020b). Melatonin improved mitochondrial membrane potential ($\Delta\psi m$), acrosomal integrity, and fertilization capacity of post/thaw sex-sorted bull sperm (Li et al., 2019). Furthermore, melatonin reduced mitochondria-derived ROS and rescued impaired penetration ability of human spermatozoa originating from mitochondrial dysfunction (Zhang et al., 2019).

In the current study, there was no significant effect of melatonin on sperm kinetics since no significant differences were found between the treatment and the sham control (ethanol) group. Nonetheless, the comparison with the control group (without any supplementation) revealed several bigger differences with regard to sperm motility parameters, incl., Total motile, Progressive motile, straight-line velocity (VSL), curvilinear velocity (VCL), average distance path (DAP), and curvilinear distance (DCL) all in favor of the treatment group. However, it was assumed that these results were not statistically different between effects stemming from melatonin or vehicle supplementation. Sperm motility, the eldest criterion for predicting male fertility (Amelar et al., 1980), is considered critical for regular fertilization (Sellem et al., 2015; Moscatelli et al., 2017).

The gene expression analysis results in the present study revealed that blastocysts derived from melatonin treated-sperm showed increased *MAPK13* mRNA expression. MAP kinase is crucial for intact male reproductive function, incl., sperm motility, hyperactivation, capacitation, and acrosome reaction (Li et al., 2009; Almog et al., 2008). The sperm acrosome reaction is Ca^{2+}-dependent and crucial for regular fertilization, which depends upon phosphorylation of the MAP kinase pathway (Kawano et al., 2010). It has been shown that bulls with high fertility had spermatozoa with lower miRNA 216b (miR-216b) concentrations but a higher level of K-RAS (Kirsten rat sarcoma viral oncogene homolog; a gene of the RAS/MAPK pathway related to cell proliferation) in zygotes and two-cell embryos with higher cleavage and higher quality blastocysts (Alves et al. (2019). Transcriptomic profiling of buffalo spermatozoa showed downregulation of 28 genes associated with the MAPK signaling pathway in bulls with low fertility compared to bulls with high fertility (Paul et al., 2021). We hypothesize that the improved fertilization ability is linked with the MAPK

pathway, which warrants future study. It was recently found that melatonin induced maturation factor (MPF) and MAPK expression in porcine oocytes, which in turn correlated with the level of GSH within oocytes, mitochondrial maturation status, and first polar body expulsion (Zhao et al., 2020).

MAP kinase signaling is critically involved in regulating early bovine embryo development, such as blastocyst formation (Madan et al. 2005), ICM cell differentiation (Negrón-Pérez et al. 2018) towards epiblast and hypoblast segregation (Kuijk et al., 2012). It is thought to play a major role in embryonic cell lineage segregation, specifically maintaining the appropriate number of hypoblast cells. The yolk sac, which develops from the hypoblast, is indispensable in early pregnancy in all mammals, playing an essential role in histotroph digestion, and is particularly critical in animals with a cotyledonary placenta to ensure proper nourishment of the fetus (Ealy et al. 2020). Growth and proliferation of the trophoblast cells, which are crucial for feto-maternal interaction during bovine placentation, were accelerated after culture in medium supplemented with epidermal growth factor (EGF), probably via activation of RAS and phosphorylation of MAP kinase (Hambruch et al. 2010).

5.2 Effects of melatonin on *in vitro* oocyte developmental competence in prepubertal donors

Little is known about the effects of melatonin on the developmental capacity of oocytes isolated from prepubertal donors. Currin et al. (2017) observed that the success of *in vitro* embryo production in prepubertal cattle after prior treatment with gonadotropin increased with the age of the animals; specifically, blastocyst rates increased, and polyspermy decreased. Results reported in Chapter II provide first evidence for a beneficial role of melatonin during IVM. We report that bovine oocyte developmental competence from prepubertal donors was significantly enhanced,

embryo development rates *in vitro* were improved as well as the proportion of expanded, hatching, and hatched blastocysts was significantly elevated after supplementing the IVM medium with melatonin. The improved embryo quality by melatonin in the present investigation was further confirmed by the increased number of cells in blastocysts, preferably in the ICM.

These results could have practical implications for the dairy industry since IVP combined with genomic selection in prepubertal heifers is increasingly being used to accelerate the genetic gain and shorten the generational interval. It is well known that embryos with a proper number of ICM cells have a higher likelihood of normal pregnancy establishment. Bovine hatched blastocysts derived from morulae with poor quality had fewer ICM cells than blastocysts grown from good quality morulae (Van Soom et al., 1997). Human embryos with an ICM with a sufficient number of tightly packed cells have been associated with a reduced risk of miscarriage (Shi et al., 2020). In the present study, it was observed that after supplementation of media with melatonin, the entire proportion of blastocysts, and specifically the number of expanded and hatching blastocysts was significantly elevated. This observation indicates improved inherent quality of the oocytes/embryos by melatonin treatment and warrants further studies to evaluate these findings *in vivo* after transfer o recipients.

During IVM of juvenile goat oocytes, melatonin supplementation decreased ROS levels, increased mitochondrial activity, ATP contents, and increased blastocyst quality reflected by an increased number of ICM cells compared to the control. Simultaneously, the expression of several developmentally important genes, incl. *ACTB*, *SLC1A1*, *SOD*, *GPx1*, *BAX*, *DNMT1*, *GCLC*, and *GDF9* was not changed in embryos produced in melatonin-supplemented medium (Soto-Heras, 2019). In adult animals, supplementation with melatonin during bovine oocyte maturation significantly up-

regulated expression of oocyte maturation associated genes (*GDF9*, *MARF1*, and *DNMT1*) and cumulus cells expansion-related genes (*PTX3*, *HAS1/2*) (Tian et al., 2014). Here, we did not investigate gene expression in oocytes/embryos produced from prepubertal animals, mainly because of a shortage of prepubertal donors.

Few data suggest that an *in vivo* treatment of prepubertal animals could have beneficial effects on oocyte/embryo development. Administration of melatonin in juvenile lambs via a subcutaneous implant improved superovulation response and the antioxidative capacity of cumulus cells probably via up-regulating mRNA expression of genes for the melatonin receptor MT1 and nuclear binding site RORα (RAR-related orphan receptor alpha) (Fang et al., 2019). Moreover, expression of several antioxidative genes (*SOD1*, *GPx4*, and *CAT*), genes involved in cumulus cells expansion (*PTX3*, *HAS2*, and *PTGS2*), *Bcl2*, the proapoptotic gene *Bax* was affected by melatonin treatment; and reduced methylation of *SOD1*, *GPx4*, and *CAT* was found (Fang et al., 2018). An *in vivo* treatment of prepubertal cattle with melatonin could be promising to further improve viable oocyte/embryo yields.

5.3 Effects of melatonin on *in vitro* embryo production: Implications for gene expression and preimplantation embryo competence *in vitro*

In Chapter IV, we observed that melatonin supplementation led to a better synchronized early embryonic development. Embryo developmental rates at days 6 and 7 were markedly enhanced when melatonin had been added to the IVP media (IVM, IVF, and IVC). Additionally, embryos derived from melatonin treatment acquired more embryonic cells, specifically TE cells, in the expanded and hatched blastocysts from day 7.5. Previously, an increased number of embryonic cells has been found in bovine embryos after adding melatonin during IVM (Tian et al., 2014; Remião et al., 2016;

Yang et al., 2017). Here, we also investigated mRNA expression of a panel of developmentally important genes to explore the effects of melatonin on *in vitro* preimplantation embryo development. We could show that melatonin had significant effects on the expression profile of the transcripts *CDH1* and *AQP3*.

Supplementation of bovine *in vitro* embryo culture medium with interferon-τ (*IFNT)* promoted bovine embryo development, as shown by higher blastocyst rates, an increased total number of embryonic cells, and upregulation of mRNA E-cadherin (*CDH1*) and connexin 43 (*GJA1*) expression (Bao et al. 2014). Targeted suppression of E-cadherin mRNA and protein by RNA interference *in vitro* resulted in lower blastocyst rates (Nganvongpanit et al., 2006), while suppression of E-cadherin expression in embryos significantly decreased the proportion of embryos that reached the blastocyst stage irrespective of *in vivo* or *in vitro* culture (Tesfaye et al., 2007).

A chemically defined medium that can perfectly mimic the physiological *in vivo* scenario would avoid the addition of serum to culture media. However, despite some progress, this remains a major challenge. Embryo culture media supplemented with bovine oviductal fluid (OF) (Lopera-Vasquez et al., 2017b; Hamdi et al., 2018), uterine fluid (UF) (Hamdi et al., 2018), bovine extracellular vesicles (EVs) (Lopera-Vásquez et al., 2017a) have been shown to improve embryonic development. Supplementation of culture media with oviductal fluid supported embryo development and produced high-quality blastocysts, as reflected by higher survival after cryopreservation, increased total cell numbers, and upregulated expression of the water channel aquaporin 3 (*AQP3*) in blastocysts (Lopera-Vasquez et al. 2017b). Blastocysts cultured in a medium supplemented with isthmic EVs showed an increased survival rate and upregulated expression of water channel *AQP3* and *DNMT3A* transcripts when compared with controls (Lopera-Vasquez et al., 2017a). Hamdi et al. (2018) observed

that blastocysts from the OF, OF+UF, and UF groups accumulated less ROS than SOF+FCS and SOF+BSA groups, while OF resulted in a better-controlled embryo methylation profile, whereas UF had increased antioxidant activity. Recent work by Qu et al. (2020) showed that melatonin is present in oviductal fluids and oviduct fluid-derived EVs. The addition of either EVs (1.87×10^{11} particles/ml) or melatonin (340 ng/ml) to culture media significantly downregulated reactive oxygen species (ROS) and increased blastocyst rates. A combined treatment of EVs (1.87 × 1010 particles/ml) and melatonin (at 34.3 pg/ml) significantly decreased the apoptosis index and increased the inner cell mass (ICM)/trophectoderm (TE) index (Qu et al. 2020). It would be promising to supplement these media (OF, UF, EV) with melatonin to further increase the efficiency. The improved embryo quality by melatonin in the current investigation could be linked to the increased expression of the transcripts for water transport *AQP3* and *CDH1*. Albeit cumulus cells have been demonstrated to produce melatonin during *in vitro* maturation (El-Raey et al., 2011; Xiao et al., 2019), our findings suggest that melatonin supplementation could be promising for improving the success rates of IVP.

5.4 Conclusions and future perspectives

The present results provide further insight into the role of melatonin during bovine *in vitro* embryo production and provide important hints to gain a better understanding of the underlying mechanisms.

Supplementation of sperm media with melatonin significantly enhanced sperm fertilization ability and embryonic development *in vitro*. Concomitantly, mRNA expression of *MAPK13* was increased, which suggests a role in preimplantation embryo cell lineage differentiation. This warrants further study into the underlying mechanisms of melatonin that regulate fertilization and preimplantation embryo development. The

activity of MAP kinase signaling in both oocyte and sperm may play a crucial role in acquiring gamete competence and may be involved in the regulation of early embryo development. The improved sperm motility/functionality may have beneficial effects on *in vitro* embryo competence and development, as observed in the current investigation. These discoveries could support the idea that sperms play a more active role in these processes than initially thought.

Bovine cumulus-oocyte complexes from prepubertal and adult dairy cattle harvested via ovum pick up and cultivated in the presence of melatonin during *in vitro* maturation showed enhanced embryo development. Embryo developmental rates almost doubled when melatonin had been incorporated into the IVP system. Analysis of embryo developmental kinetics revealed that oocytes treated with melatonin led to an increased proportion of expanded and hatching blastocysts (i.e., advanced development). We provide here the first evidence for improved oocyte competence from prepubertal bovine donors by melatonin *in vitro*. Future studies that include embryo transfers to recipients are needed to substantiate this finding.

Finally, we showed that melatonin improved bovine *in vitro* embryo development and led to a better synchronized early embryo development, specifically compaction and blastocyst formation. Moreover, for the first time, we could demonstrate that transcripts crucial for early development, such as type 1 epithelial cadherin 1 (*CDH1*), mitogen-Activated Protein Kinase 13 (*MAPK13*), and the ion exchange and water transport gene aquaporin 3 (*AQP3*), are modulated by melatonin. This provides a solid basis for future studies into the underlying mechanism of melatonin in oocyte and embryo development.

Collectively, these findings contribute towards a better understanding of the underlying mechanisms of melatonin in bovine oocyte and embryo developmental competence and provide a new basis for future studies in this area. We also show for the first time that dissolving melatonin with ethanol could eventually impair embryo development, as shown by alterations of embryonic cell counting and altered expression profiles of *ATP1A1*, *CDH1*, *CDX2*, and *MAPK13* in the sham control group, suggesting that less harmful vehicles for dissolving melatonin need to be explored.

6. References

Acuña-Castroviejo, D., Escames, G., Venegas, C., Díaz-Casado, M.E., Lima-Cabello, E., López L.C., Rosales-Corral, S., Tan, D.X., Reiter, R.J. (2014). Extrapineal melatonin: sources, regulation, and potential functions. Cell Mol Life Sci. **71**(16), 2997-3025.

Agarwal, A., Gupta, S., Sharma, R.K. (2005). Role of oxidative stress in female reproduction. Reprod Biol Endocrino. **3**:28.

Alegre, L., Del Gallego, R., Arrones, S., Hernández, P., Muñoz, M., Meseguer, M. (2019). Novel noninvasive embryo selection algorithm combining time-lapse morphokinetics and oxidative status of the spent embryo culture medium. Fertil Steril. **111**(5), 918-927.

Al-Gubory, K.H., Bolifraud, P., Germain, G., Nicole, A., Ceballos-Picot, I. (2004). Antioxidant enzymatic defence systems in sheep corpus luteum throughout pregnancy. Reproduction. **128**(6), 767-774.

Al-Gubory, K.H., Garrel, C., Faure, P., Sugino, N. (2014). Roles of antioxidant enzymes in corpus luteum rescue from reactive oxygen species-induced oxidative stress. Reprod Biomed Online. **25**(6), 551-560.

Almog, T., Lazar, S., Reiss, N., Etkovitz, N., Milch, E., Rahamim, N., Dobkin-Bekman, M., Rotem, R., Kalina, M., Ramon, J., Raziel, A., Breitbart, H., Seger, R., Naor, Z. (2008). Identification of extracellular signal-regulated kinase 1/2 and p38 MAPK as regulators of human sperm motility and acrosome reaction and as predictors of poor spermatozoan quality. J Biol Chem. **283**(21), 14479-89.

Alves, M.B.R., de Arruda, R.P., De Bem, T.H.C., Florez-Rodriguez, S.A., Sá Filho, M.F., Belleannée, C., Meirelles, F.V., da Silveira, J.C., Perecin, F., Celeghini, E.C.C. (2019). Sperm-borne miR-216b modulates cell proliferation during early embryo development via K-RAS. Sci Rep. **9**(1):10358.

Amelar, R.D., Dubin, L., Schoenfeld, C. (1980). Sperm motility. Fertil Steril. **34**(3), 197-215.

Antonsson, B. (2001). Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. Cell Tissue Res. **306**(3), 347-361.

Armstrong, S., Bhide, P., Jordan, V., Pacey, A., Farquhar, C. (2018). Time-lapse systems for embryo incubation and assessment in assisted reproduction. Cochrane Database Syst Rev. **5**(5):CD011320.

Aroor, AR, Shukla SD (2004). MAP kinase signaling in diverse effects of ethanol. Life Sci **74**(19), 2339-64.

Aydiner, F., Yetkin, C.E., Seli, E. (2010). Perspectives on emerging biomarkers for non-invasive assessment of embryo viability in assisted reproduction. Curr Mol Med. **10**(2), 206-215.

Bahrami, M., Morris, M.B., Day, M.L. (2019). Amino acid supplementation of a simple inorganic salt solution supports efficient in vitro maturation (IVM) of bovine oocytes. Sci Rep. **9**(1), 11739.

Bain, N.T., Madan, P., Betts, D.H. (2011). The early embryo response to intracellular reactive oxygen species is developmentally regulated. Reprod Fertil Dev. **23**(4), 561-575.

Baltz, J.M. (2012). Media composition: salts and osmolality. Methods Mol Biol **912**, 61-80.

Bansal, A.K., Bilaspuri, G.S. (2010). Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int. 2010:686137.

Bao, Z.J., Zhao, S., Haq, I.U., Zeng, S.M. (2014). Recombinant bovine interferon-τ enhances in vitro development of bovine embryos by upregulating expression of connexin 43 and E-cadherin. J Dairy Sci. **97**(11), 6917-6925.

Barcroft, L.C., Gill, S.E., Watson, A.J. (2002). The gamma-subunit of the Na-K-ATPase as a potential regulator of apical and basolateral Na+-pump isozymes during development of bovine pre-attachment embryos. Reproduction. **124**(3), 387-97.

Barcroft, L.C., Offenberg, H., Thomsen, P., Watson, A.J. (2003). Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation. Dev Biol. **256**(2), 342-354.

Belhad, Slimen, I., Najar, T., Ghram, A., Abdrrabba, M. (2016). Heat stress effects on livestock: molecular, cellular and metabolic aspects, a review. J Anim Physiol Anim Nutr (Berl). **100**(3), 401-412.

Bell CE, Calder MD, Watson AJ (2008). Genomic RNA profiling and the programme controlling preimplantation mammalian development. Mol Hum Reprod. **14**(12), 691-701.

Berg, D.A., Menino, A.R. Jr. (1992). Bovine embryos produce a urokinase-type plasminogen activator. Mol Reprod Dev. **31**(1), 14-19.

Bernal-Ulloa, S.M., Heinzmann, J., Herrmann, D., Hadeler, K.G., Aldag, P., Winkler, S., Pache, D., Baulain, U., Lucas-Hahn, A., Niemann, H. (2016). Cyclic AMP Affects Oocyte Maturation and Embryo Development in Prepubertal and Adult Cattle. PLoS One. **11**(2):e0150264.

Bertolini, M., Beam, S.W., Shim, H., Bertolini, L.R., Moyer, A.L., Famula, T.R., Anderson, G.B. (2002). Growth, development, and gene expression by in vivo- and in vitro-produced day 7 and 16 bovine embryos. Mol Reprod Dev. **63**(3), 318-328.

Betteridge, K.J., Flechon, J.E. (1988). The anatomy and physiology of pre- attachement bovine embryos. Theriogenology. **29,** 155-187.

Betts, D.H., Barcroft, L.C., Watson, A.J. (1998). Na/K-ATPase-mediated 86Rb+ uptake and asymmetrical trophectoderm localization of alpha1 and alpha3 Na/K-ATPase isoforms during bovine preattachment development. Dev Biol. **197**(1), 77-92.

Bó, G.A., Mapletoft, R.J. (2013). Evaluation and classification of bovine embryos. Anim Reprod. **10**(3), 344-348.

Bols, P.E.J., Ysabaert, M.T., Van Soom, A. de Kruif, A. (1997). Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact cumulus oocyte complexes. Theriogenolgy. **47**(6), 1221-1236.

Bonnefont-Rousselot, D., Collin, F. (2010). Melatonin: action as antioxidant and potential applications in human disease and aging. Toxicology. **278**(1), 55-67.

Bubenik, G.A. (1980). Localization of melatonin in the digestive tract of the rat. Effect of maturation, diurnal variation, melatonin treatment and pinealectomy. Horm Res. **12**(6), 313-323.

Burton, G.J., Hempstock, J., Jauniaux, E. (2003). Oxygen, early embryonic metabolism and free radical-mediated embryopathies. Reprod Biomed Online. **6**(1), 84-96.

Callesen, H., Greve, T., Christensen, F. (1987). Ultrasonically guided aspiration of bovine follicular oocytes. Theriogenology. **27**(1): 217 (Abstract).

Camargo LS, Viana JH, Sá WF, Ferreira AM, Vale Filho VR., 2005. Developmental competence of oocytes from prepubertal Bos indicus crossbred cattle. Anim Reprod Sci. **85**(1-2), 53-59.

Cardinali, D.P., Nagle, C.A., Rosner, J.M. (1974). Periodic changes in rat retinal and pineal melatonin synthesis. Acta Physiol Lat Am. **24**(1), 97-8.

Carlomagno, G., Minini, M., Tilotta, M., Unfer, V., (2018). From Implantation to Birth: Insight into Molecular Melatonin Functions. Int J Mol Sci. **19**(9), 2802.

Carrillo-Vico, A., Calvo, J.R., Abreu, P., Lardone, P.J., García-Mauriño, S., Reiter, R.J., Guerrero, J.M. (2004). Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance. FASEB J. **18**(3), 537-539.

Carrillo-Vico, A., Lardone, P.J., Alvarez-Sánchez, N., Rodríguez-Rodríguez, A., Guerrero, J.M. (2013). Melatonin: buffering the immune system. Int J Mol Sci. **14**(4), 8638-8683.

Cetica, P.D., Pintos, L.N., Dalvit, G.C., Beconi, M.T. (2001). Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. IUBMB Life. **51**(1), 57-64.

Currin, L., Michalovic, L., Bellefleur, A.M., Gutierrez, K., Glanzner, W., Schuermann, Y., Bohrer, R.C., Dicks, N., da Rosa, P.R., De Cesaro, M.P., Lopez, R., Grand, F.X., Vigneault, C., Blondin, P., Gourdon, J., Baldassarre, H., Bordignon, V. (2017). The effect of age and length of gonadotropin stimulation on the in vitro embryo development of Holstein calf oocytes. Theriogenology. **104**, 87-93.

Daigneault, B.W, Rajput, S., Smith, G.W., Ross, P.J. (2018). Embryonic POU5F1 is Required for Expanded Bovine Blastocyst Formation. Sci Rep. **8**(1), 7753.

Damiani, P., Fissore, R.A., Cibelli, J.B., Long, C.R., Balise, J.J., Robl, J.M., Duby, R.T. (1996). Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. Mol Reprod Dev. **45**(4), 521-534.

de Loos F.A., Bevers, M.M., Dieleman, S.J., Kruip, T.A. (1991). Morphology of preovulatory bovine follicles as related to oocyte maturation. Theriogenology. **35**(3), 527-535.

de Loos, F.A., van Vliet, C., van Maurik, P., Kruip, T.A. (1989). Morphology of immature bovine oocytes. Gamete Res. **24**(2), 197-204.

de Matos, D.G., Furnus, C.C. (2000). The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. Theriogenology. **53**(3), 761-771.

De Vos, M., Smitz, J., Thompson, J.G., Gilchrist, R.B. (2016). The definition of IVM is clear-variations need defining. Hum Reprod. **31**(11), 2411-2415.

Deleuze, S., Goudet, G. (2010). Cysteamine supplementation of in vitro maturation media: a review. Reprod Domest Anim. **45**(6):e476-82.

Denault, J.B., Salvesen, GS. (2008). Apoptotic caspase activation and activity. Methods Mol Biol. **414**, 191-220.

Dennery, P.A. (2007). Effects of oxidative stress on embryonic development. Birth Defects Res C Embryo Today. **81**(3), 155-162.

Deutsch DR, Fröhlich T, Otte KA, Beck A, Habermann FA, Wolf E, Arnold GJ., 2014. Stage-specific proteome signatures in early bovine embryo development. J Proteome Res. **13**(10), 4363-4376.

Diederich, M., Hansmann, T., Heinzmann, J., Barg-Kues, B., Herrmann, D., Aldag, P., Baulain, U., Reinhard, R., Kues, W., Weissgerber, C., Haaf, T., Niemann, H. (2012). DNA methylation and mRNA expression profiles in bovine oocytes derived from prepubertal and adult donors. Reproduction. **144**(3), 319-330.

Dirvanauskas, D., Maskeliunas, R., Raudonis, V., Damasevicius, R. (2019). Embryo development stage prediction algorithm for automated time lapse incubators. Comput Methods Programs Biomed. **177,** 161-174.

Ealy, A.D., Wooldridge, L.K., McCoski, S.R. (2019). BOARD INVITED REVIEW: Post-transfer consequences of in vitro-produced embryos in cattle. J Anim Sci. **97**(6), 2555-2568.

Edwards, R.G. (1962). Meiosis in ovarian oocytes of adult mammals. Nature. **196**:446-450.

El-Raey, M., Geshi, M., Somfai, T., Kaneda, M., Hirako, M., Abdel-Ghaffar, A.E., Sosa, G.A., El-Roos, M.E., Nagai, T. (2011). Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. Mol Reprod Dev. **78**(4), 250-262.

Emanuelli, I.P., Costa, C.B., Rafagnin, Marinho, L.S., Seneda, M.M., Meirelles, F.V. (2019). Cumulus-oocyte interactions and programmed cell death in bovine embryos produced in vitro. Theriogenology. **126**, 81-87.

Erbach, G.T., Lawitts, J.A., Papaioannou, V.E., Biggers, J.D. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. Biol Reprod. **50**(5), 1027-1033.

Espino, J., Macedo, M., Lozano, G., Ortiz, Á., Rodríguez, C., Rodríguez, A.B., Bejarano, I. (2019). Impact of Melatonin Supplementation in Women with Unexplained Infertility Undergoing Fertility Treatment. Antioxidants (Basel). **8**(9), 338.

Fang, Y., Deng, S., Zhang, J., Liu, H., Li, Y., Zhang, X., Liu, Y. (2018). Melatonin-Mediated Development of Ovine Cumulus Cells, Perhaps by Regulation of DNA Methylation. Molecules. **23**(2), 494.

Fang, Y., Zhang, J., Li, Y., Guo, X., Li, J., Zhong, R., Zhang, X. (2019). Melatonin-induced demethylation of antioxidant genes increases antioxidant capacity through RORα in cumulus cells of prepubertal lambs. Free Radic Biol Med. **131**, 173-183.

Fang, Y., Zhao, C., Xiang, H., Jia, G., Zhong, R. (2020a). Melatonin improves cryopreservation of ram sperm by inhibiting mitochondrial permeability transition pore opening. Reprod Domest Anim. **55**(9), 1240-1249.

Fang, Y., Zhao, C., Xiang, H., Zhao, X., Zhong, R. (2020b). Melatonin Inhibits Formation of Mitochondrial Permeability Transition Pores and Improves Oxidative Phosphorylation of Frozen-Thawed Ram Sperm. Front Endocrinol (Lausanne). **10**, 896.

Ferré, L.B., Kjelland, M.E., Strøbech, L.B., Hyttel, P., Mermillod, P., Ross, P.J. (2020). Review: Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. Animal. **14**(5), 991-1004.

Fuchs Weizman, N., Wyse, B.A., Antes, R., Ibarrientos, Z., Sangaralingam, M., Motamedi, G., Kuznyetsov, V., Madjunkova, S., Librach, C.L. (2019). Towards Improving Embryo Prioritization: Parallel Next Generation Sequencing of DNA and RNA from a Single Trophectoderm Biopsy. Sci Rep. **9**(1), 2853.

Galano, A., Tan, D.X., Reiter, R.J. (2013). On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. J Pineal Res. **54**(3), 245-257.

Gandhi, A.P., Lane, M., Gardner, D.K., Krisher, R.L. (2000). A single medium supports development of bovine embryos throughout maturation, fertilization and culture. Hum Reprod. **15**(2), 395-401.

Gandolfi, F., Luciano, A.M., Modina, S., Ponzini, A., Pocar, P., Armstrong, D.T., Lauria, A. (1997). The in vitro developmental competence of bovine oocytes can be related to the morphology of the ovary. Theriogenology. **48**(7), 1153-1160.

Gleicher, N., Friberg, J., Fullan, N., Giglia, R.V., Mayden, K., Kesky, T., Siegel, I. (1983). EGG retrieval for in vitro fertilisation by sonographically controlled vaginal culdocentesis. Lancet. **2**(8348), 508-9.

Goissis, M.D., Cibelli, J.B. (2014). Functional characterization of CDX2 during bovine preimplantation development in vitro. Mol Reprod Dev. **81**(10), 962-970.

Gordon, I. R. (2003). Laboratory production of cattle embryos, Biotechnology in agriculture series, N° 27, (2nd ed). (CAB International, Wallingford, U.K). Pp. 220-270.

Goud, A.P., Goud, P.T., Diamond, M.P., Gonik, B., Abu-Soud, H.M. (2008). Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid. Free Radic Biol Med. **44**(7), 1295-1304.

Greenfeld, C.R., Pepling, M.E., Babus, J.K., Furth, P.A., Flaws, J.A. (2007). BAX regulates follicular endowment in mice. Reproduction. **133**(5), 865-876.

Guérin, .P, El Mouatassim, S., Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update. **7**(2), 75-89.

Gutiérrez-Añez, J.C., Lucas-Hahn, A., Hadeler, K.G., Aldag, P., Niemann H. (2021). Melatonin enhances in vitro developmental competence of cumulus-oocyte complexes collected by ovum pick-up in prepubertal and adult dairy cattle. Theriogenology. **161**, 285-293.

Hambruch, N., Haeger, J.D., Dilly, M., Pfarrer, C. (2010). EGF stimulates proliferation in the bovine placental trophoblast cell line F3 via Ras and MAPK. Placenta. **31**(1), 67-74.

Hamdi, M., Lopera-Vasquez, R., Maillo, V., Sanchez-Calabuig, M.J., Núnez, C., Gutierrez-Adan, A., Rizos, D. (2018). Bovine oviductal and uterine fluid support in vitro embryo development. Reprod Fertil Dev. **30**(7), 935-945.

Hardeland, R. (2018). Melatonin and inflammation-Story of a double-edged blade. J Pineal Res. **65**(4), e12525.

Hardeland, R. (2019). Aging, Melatonin, and the Pro- and Anti-Inflammatory Networks. Int J Mol Sci. **20**(5), 1223.

Harvey, A.J. (2019). Mitochondria in early development: linking the microenvironment, metabolism and the epigenome. Reproduction. **157**(5), R159-R179.

Hoffman, R.A., Reiter, R.J. (1965). Pineal gland: influence on gonads of male hamsters. Science. **148**(3677), 1609-1611.

Houghton, F.D., Humpherson, P.G., Hawkhead, J.A., Hall, C.J., Leese, H.J. (2003). Na+, K+, ATPase activity in the human and bovine preimplantation embryo. Dev Biol. **263**(2), 360-366.

Hyttel, P., Greve, T., Callesen, H. (1989). Ultrastructural aspects of oocyte maturation and fertilization in cattle. J Reprod Fertil Suppl. **38**, 35-47.

Ikeda, S., Imai, H., Yamada, M. (2003). Apoptosis in cumulus cells during in vitro maturation of bovine cumulus-enclosed oocytes. Reproduction. **125**(3), 369-376.

Itoh, M.T., Ishizuka, B., Kudo, Y., Fusama, S., Amemiya, A., Sumi, Y. (1997). Detection of melatonin and serotonin N-acetyltransferase and hydroxyindole-O-methyltransferase activities in rat ovary. Mol Cell Endocrinol. **136**(1), 7-13.

Jiang, W., Li, Y., Zhao, Y., Gao, Q., Jin, Q., Yan, C., Xu, Y. (2020). l-carnitine supplementation during in vitro culture regulates oxidative stress in embryos from bovine aged oocytes. Theriogenology. **143**, 64-73.

Johnson, M.H., McConnell, J.M. (2004). Lineage allocation and cell polarity during mouse embryogenesis. Semin Cell Dev Biol. **15**(5), 583-597.

Kang, J.T., Koo, O.J., Kwon, D.K., Park, H.J., Jang, G., Kang, S.K., Lee, B.C. (2009). Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. J Pineal Res. **46**(1), 22-28.

Katz-Jaffe, M.G., Gardner, D.K., Schoolcraft, W.B. (2006). Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability. Fertil Steril. **85**(1), 101-107.

Kawano, N., Ito, J., Kashiwazaki, N., Yoshida, M. (2010). Phosphorylation of the MAPK pathway has an essential role in the acrosome reaction in miniature pig sperm. Reprod Domest Anim. **45**(2), 263-268.

Khalil, W.A., Marei, W.F., Khalid, M. (2013). Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development. Theriogenology. **80**(2), 161-168.

Khurana, N.K., Niemann, H. (2000). Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. Biol Reprod. **62**(4), 847-856.

Kidder, G.M., Watson, A.J. (2005). Roles of Na,K-ATPase in early development and trophectoderm differentiation. Semin Nephrol. **25**(5), 352-355.

Kim, E.H., Kim, G.A., Taweechaipaisankul, A., Lee, S.H., Qasim, M., Ahn, C., Lee, B.C. (2019). Melatonin enhances porcine embryo development via the Nrf2/ARE signaling pathway. J Mol Endocrinol. **63**(3), 175-185.

Komninou, E.R., Remião, M.H., Lucas, C.G., Domingues, W.B., Basso, A.C., Jornada, D.S., Deschamps, J.C., Beck, R.C., Pohlmann, A.R., Bordignon, V., Seixas, F.K., Campos, V.F., Guterres, S.S., Collares, T. (2016). Effects of Two Types of Melatonin-Loaded Nanocapsules with Distinct Supramolecular Structures: Polymeric (NC) and Lipid-Core Nanocapsules (LNC) on Bovine Embryo Culture Model. PLoS One. **11**(6), e0157561.

Kratz, E.M., Piwowar, A. (2017). Melatonin, advanced oxidation protein products and total antioxidant capacity as seminal parameters of prooxidant-antioxidant balance and their connection with expression of metalloproteinases in context of male fertility. J Physiol Pharmacol. **68**(5), 659-668.

Kruip, T.A., Dieleman, S.J. (1982). Macroscopic classification of bovine follicles and its validation by micromorphological and steroid biochemical procedures. Reprod Nutr Dev. **22**(3), 465-473.

Kuijk, E.W., van Tol, L.T., Van de Velde, H., Wubbolts, R., Welling, M., Geijsen, N., Roelen, B.A. (2012). The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development. **139**(5), 871-882.

Lazzari, G., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H., Galli, C. (2002). Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod. **67**(3), 767-775.

Lerner, A.B., Case, J.D., Takahashi, Y., Lee, T.H., Mori, W. (1958). Isolation of melatonin, the pineal factor that lightens melanocytes. J Am Chem Soc. **80**, 2587- 2592.

Li, C.Y., Hao, H.S., Zhao, Y.H., Zhang, P.P., Wang, H.Y., Pang, Y.W., Du, W.H., Zhao, S.J., Liu, Y., Huang, J.M., Wang, J.J., Ruan, W.M., Hao, T., Reiter, R.J., Zhu, H.B., Zhao. X.M. (2019). Melatonin Improves the Fertilization Capacity of Sex-Sorted Bull Sperm by Inhibiting Apoptosis and Increasing Fertilization Capacitation via MT1. Int J Mol Sci. **20**(16), 3921.

Li, M.W., Mruk, D.D., Cheng, C.Y. (2009). Mitogen-activated protein kinases in male reproductive function. Trends Mol Med. **15**(4), 159-168.

Lonergan, P. (2007). State-of-the-art embryo technologies in cattle. Soc Reprod Fertil Suppl. **64**, 315-325.

Lopera-Vasquez, R., Hamdi, M., Maillo, V., Gutierrez-Adan, A., Bermejo-Alvarez, P., Ramírez, M.Á., Yáñez-Mó, M., Rizos, D. (2017a). Effect of bovine oviductal extracellular vesicles on embryo development and quality in vitro. Reproduction. **153**(4), 461-470.

Lopera-Vasquez, R., Hamdi, M., Maillo, V., Lloreda, V., Coy, P., Gutierrez-Adan, A., Bermejo-Alvarez, P., Rizos, D. (2017b). Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. Reprod Fertil Dev. **29**(3), 621-629.

Loren, P., Sánchez, R., Arias, M.E., Felmer, R., Risopatrón, J., Cheuquemán, C. (2017). Melatonin Scavenger Properties against Oxidative and Nitrosative Stress: Impact on Gamete Handling and In Vitro Embryo Production in Humans and Other Mammals. Int J Mol Sci. **18**(6), 1119.

Lü, J.M., Lin, P.H., Yao, Q., Chen, C. (2010). Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. J Cell Mol Med. **14**(4), 840-860.

Luvoni, G.C., Keskintepe, L., Brackett, B.G. (1996). Improvement in bovine embryo production in vitro by glutathione-containing culture media. Mol Reprod Dev. **43**(4), 437-443.

MacPhee, D.J., Jones, D.H., Barr, K.J., Betts, D.H., Watson, A.J., Kidder, G.M. (2000). Differential involvement of Na(+),K(+)-ATPase isozymes in preimplantation development of the mouse. Dev Biol. **222**(2), 486-498.

Madan, P., Calder, M.D., Watson, A.J. (2005). Mitogen-activated protein kinase (MAPK) blockade of bovine preimplantation embryogenesis requires inhibition of both p38 and extracellular signal-regulated kinase (ERK) pathways. Reproduction. **130**(1), 41-51.

Madan, P., Rose, K., Watson, A.J. (2007). Na/K-ATPase beta1 subunit expression is required for blastocyst formation and normal assembly of trophectoderm tight junction-associated proteins. J Biol Chem. **282**(16), 12127-12134.

Madrid Gaviria, S., López Herrera, A., Urrego, R., Restrepo Betancur, G., Echeverri Zuluaga, J.J. (2019). Effect of resveratrol on vitrified in vitro produced bovine embryos: Recovering the initial quality. Cryobiology. **89**, 42-50.

Maekawa, M., Yamamoto, T., Tanoue, T., Yuasa, Y., Chisaka, O., Nishida, E. (2005). Requirement of the MAP kinase signaling pathways for mouse preimplantation development. Development. **132**(8), 1773-1783.

Maître, J.L., Niwayama, R., Turlier, H., Nédélec, F., Hiiragi, T. (2015). Pulsatile cell-autonomous contractility drives compaction in the mouse embryo. Nat Cell Biol. **17**(7), 849-855.

Marques, A., Santos, P., Antunes, G., Chaveiro, A., Moreira da Silva, F. (2010). Effect of alpha-tocopherol on bovine in vitro fertilization. Reprod Domest Anim. **45**(1), 81-85.

Marques, T.C., da Silva Santos, E.C., Diesel, T.O., Leme, L.O., Martins, C.F., Dode, M., Alves, B.G., Costa, F., de Oliveira, E.B., Gambarini, M.L. (2018). Melatonin reduces apoptotic cells, SOD2 and HSPB1 and improves the in vitro production and quality of bovine blastocysts. Reprod Domest Anim. **53**(1), 226-236.

Martinou, J.C., Green, D.R. (2001). Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol. **2**(1), 63-67.

McCord, C.P., Allen, F.P. (1917). Evidences associating pineal gland function with alterations in pigmentation. J Exp Zool. **23**, 207-224.

Meng,W., Takeichi, M. (2009). Adherens junction: molecular architecture and regulation. Cold Spring Harb Perspect Biol. **1**(6), a002899.

Merton, J.S., Knijn, H.M., Flapper, H., Dotinga, F., Roelen, B.A., Vos, P.L., Mullaart, E. (2013). Cysteamine supplementation during in vitro maturation of slaughterhouse- and opu-derived bovine oocytes improves embryonic development without affecting cryotolerance, pregnancy rate, and calf characteristics. Theriogenology. **80**(4), 365-371.

Michalovic, L., Currin, L., Gutierrez, K., Bellefleur, A.M., Glanzner, W.G., Schuermann, Y., de Macedo, M.P., Bohrer, R.C., Dicks, N., Lopez, R., Taibi, M., Madogwe, E., St-Yves, A., Mondadori, R.G., Gourdon, J., Vigneault, C., Baldassarre, H., Bordignon, V. (2018). Granulosa cells of prepubertal cattle respond to gonadotropin signaling and upregulate genes that promote follicular growth and prevent cell apoptosis. Mol Reprod Dev. **85**(12), 909-920.

Mishra, A., Seshagiri, P.B. (2000). Evidence for the involvement of a species-specific embryonic protease in zona escape of hamster blastocysts. Mol Hum Reprod. **6**(11), 1005-1012.

Morris, D., Grealy, M., Leese, H., Diskin, M., Sreenan, J. (2001). Cattle embryo growth, development and viability. Beef Prod Ser. **36,** 1- 32. Available from: https://www.researchgate.net/publication/242166058_Cattle_embryo_growth_development_and_viability [accessed Feb 11 2021].

Morris, S.A., Zernicka-Goetz, M. (2012). Formation of distinct cell types in the mouse blastocyst. Results Probl Cell Differ. **55**, 203-217.

Mortezaee, K., Potes, Y., Mirtavoos-Mahyari, H., Motevaseli, E., Shabeeb, D., Musa, A.E., Najafi, M., Farhood, B. (2019). Boosting immune system against cancer by melatonin: A mechanistic viewpoint. Life Sci. **238**, 116960.

Moscatelli, N., Spagnolo, B., Pisanello, M., Lemma, E.D., De Vittorio, M., Zara, V., Pisanello, F., Ferramosca, A. (2017). Single-cell-based evaluation of sperm progressive motility via fluorescent assessment of mitochondria membrane potential. Sci Rep. **7**(1), 17931.

Nagina, G., Asima, A., Nemat, U., Shamim, A. (2016). Effect of melatonin on maturation capacity and fertilization of Nili-Ravi buffalo (Bubalus bubalis) oocytes. Open Vet J. **6**(2), 128-134.

Natale, D.R., Paliga, A.J., Beier, F., D'Souza, S.J., Watson, A.J. (2004). p38 MAPK signaling during murine preimplantation development. Dev Biol. **268**(1), 76-88.

Negrón-Pérez, V.M. (2017). Differentiation and development of the bovine blastocyst [PhD Thesis]. University of Florida. 213 p.

Negrón-Pérez, V.M., Hansen, P.J. Role of yes-associated protein 1, angiomotin, and mitogen-activated kinase kinase 1/2 in development of the bovine blastocyst. Biol Reprod. **98**(2), 170-183.

Nganvongpanit, K., Müller, H., Rings, F., Gilles, M., Jennen, D., Hölker, M., Tholen, E., Schellander, K., Tesfaye, D. (2006). Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. Mol Reprod Dev. **73**(2), 153-163.

Niles, L.P., Wang, J., Shen, L., Lobb, D.K., Younglai, E.V. (1999). Melatonin receptor mRNA expression in human granulosa cells. Mol Cell Endocrinol. **156**(1-2), 107-110.

Oropeza, A., Wrenzycki, C., Herrmann, D., Hadeler, K.G., Niemann, H. (2004). Improvement of the developmental capacity of oocytes from prepubertal cattle by intraovarian insulin-like growth factor-I application. Biol Reprod. **70**(6), 1634-1643.

Ortega, M.S., Kelleher, A.M., O'Neil, E., Benne, J., Cecil, R., Spencer, T.E. (2020). NANOG is required to form the epiblast and maintain pluripotency in the bovine embryo. Mol Reprod Dev. **87**(1), 152-160.

Ortiz, A., Espino, J., Bejarano, I., Lozano, G.M., Monllor, F., García, J.F., Pariente, J.A., Rodríguez, A.B. (2011). High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. J Pineal Res. **50**(2), 132-139.

Overström, E. (1996). In vitro assessment of embryo viability. Theriogenology. **45**, 3-16.

Palma, G.A. (2008). Biotecnología de la Reproduccion. 2da ed. – Mar de Plata. 669 p.

Palma, G.A., Tortonese, D.J., Sinowatz, F. (2001). Developmental capacity in vitro of prepubertal oocytes. Anat Histol Embryol. **30**(5), 295-300.

Pang, S.F., Brown, G.M., Grota, L.J., Chambers, J.W., Rodman, R.L. (1977). Determination of N-acetylserotonin and melatonin activities in the pineal gland, retina, harderian gland, brain and serum of rats and chickens. Neuroendocrinology. **23**(1), 1-13.

Pang, Y.W., Sun, Y.Q., Jiang, X.L., Huang, Z.Q., Zhao, S.J., Du, W.H., Hao, H.S., Zhao, X.M., Zhu, H.B. (2016). Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. Mol Reprod Dev. **83**(11), 993-1002.

Parrish, J.J. (2014). Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. Theriogenology. **81**(1), 67-73.

Parrish, J.J., Foote, R.H. (1987). Quantification of bovine sperm separation by a swim-up method. Relationship to sperm motility, integrity of acrosomes, sperm migration in polyacrylamide gel and fertility. J Androl. **8**(4), 259-266.

Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H., First, N.L. (1986). Bovine in vitro fertilization with frozen-thawed semen. Theriogenology. **25**(4), 591-600.

Paul, N., Kumaresan, A., Das Gupta, M., Nag, P., Guvvala, P.R., Kuntareddi, C., Sharma, A., Selvaraju, S., Datta, T.K. (2021). Transcriptomic Profiling of Buffalo Spermatozoa Reveals Dysregulation of Functionally Relevant mRNAs in Low-Fertile Bulls. Front Vet Sci. **7**, 609518.

Pesce, M., De Felici, M. (1994). Apoptosis in mouse primordial germ cells: a study by transmission and scanning electron microscope. Anat Embryol (Berl). **189**(5), 435-440.

Pieterse, M.C., Kappen, K.A., Kruip, T.A., Taverne, M.A. (1988). Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology. **30**(4), 751-762.

Presicce, G.A., Jiang, S., Simkin, M., Zhang, L., Looney, C.R., Godke, R.A., Yang, X. (1997). Age and hormonal dependence of acquisition of oocyte competence for embryogenesis in prepubertal calves. Biol Reprod. **56**(2), 386-392.

Qu, P., Luo, S., Du, Y., Zhang, Y., Song, X., Yuan, X., Lin, Z., Li, Y., Liu, E. (2020). Extracellular vesicles and melatonin benefit embryonic develop by regulating reactive oxygen species and 5-methylcytosine. J Pineal Res. **68**(3), e12635.

Reiter, R.J., Richardson, B.A., Matthews, S.A., Lane, S.J., Ferguson, B.N. (1983). Rhythms in immunoreactive melatonin in the retina and Harderian gland of rats: persistence after pinealectomy. Life Sci. **32**(11), 1229-1236.

Reiter, R.J., Rosales-Corral, S., Tan, D.X., Jou, M.J., Galano, A., Xu, B. (2017a). Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. Cell Mol Life Sci. **274**(21), 3863-3881.

Reiter, R.J., Rosales-Corral, S.A., Manchester, L.C., Tan, D.X. (2013a). Peripheral reproductive organ health and melatonin: ready for prime time. Int J Mol Sci. 2013a **14**(4), 7231-7272.

Reiter, R.J., Rosales-Corral, S.A., Tan, D.X., Acuna-Castroviejo, D., Qin, L., Yang, S.F., Xu, K. (2017b). Melatonin, a Full Service Anti-Cancer Agent: Inhibition of Initiation, Progression and Metastasis. Int J Mol Sci. **18**(4), 843.

Reiter, R.J., Tan, D.X., Rosales-Corral, S., Galano, A., Zhou, X.J., Xu, B. (2018). Mitochondria: Central Organelles for Melatonin's Antioxidant and Anti-Aging Actions. Molecules. **23**(2):509.

Reiter, R.J., Tan, D.X., Rosales-Corral, S., Manchester, L.C. (2013b). The universal nature, unequal distribution and antioxidant functions of melatonin and its derivatives. Mini Rev Med Chem. **13**(3), 373-384.

Remião, M.H., Lucas, C.G., Domingues, W.B., Silveira, T., Barther, N.N., Komninou, E.R., Basso, A.C., Jornada, D.S., Beck, R.C., Pohlmann, A.R., Junior, A.S., Seixas, F.K., Campos, V.F., Guterres, S.S., Collares, T. (2016). Melatonin delivery by nanocapsules during in vitro bovine oocyte maturation decreased the reactive oxygen species of oocytes and embryos. Reprod Toxicol. **63**, 70-81.

Reyes, J.M., Ross, P.J. (2016). Cytoplasmic polyadenylation in mammalian oocyte maturation. Wiley Interdiscip Rev RNA. **7**(1), 71-89.

Rocha, C.S., Rato, L., Martins, A.D., Alves, M.G., Oliveira. P.F. (2015). Melatonin and male reproductive health: relevance of darkness and antioxidant properties. Curr Mol Med. **15**(4), 299-311.

Rocha-Frigoni, N.A., Leão, B.C., Dall'Acqua, P.C., Mingoti, G.Z. (2016). Improving the cytoplasmic maturation of bovine oocytes matured in vitro with intracellular and/or extracellular antioxidants is not associated with increased rates of embryo development. Theriogenology. **86**(8), 1897-1905.

Rosenkrans, C.F. Jr., First, N.L. (1994). Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J Anim Sci. **72**(2), 434-437.

Roth, Z. (2017). Effect of Heat Stress on Reproduction in Dairy Cows: Insights into the Cellular and Molecular Responses of the Oocyte. Annu Rev Anim Biosci. **5**, 151-170.

Rucker, E.B. 3rd., Dierisseau, P., Wagner, K.U., Garrett, L., Wynshaw-Boris, A., Flaws, J.A., Hennighausen, L. (2000). Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. Mol Endocrinol. **14**(7), 1038-1052.

Sagan, L. (1967). On the origin of mitosing cells. J Theor Biol. **14**(3), 255-274.

Sakurai, N., Takahashi, K., Emura, N., Fujii, T., Hirayama, H., Kageyama, S., Hashizume, T., Sawai, K. (2016). The Necessity of OCT-4 and CDX2 for Early Development and Gene Expression Involved in Differentiation of Inner Cell Mass and Trophectoderm Lineages in Bovine Embryos. Cell Reprogram. **18**(5), 309-318.

Salamone, D.F., Damiani, P., Fissore, R.A., Robl, J.M., Duby, R.T. (2001). Biochemical and developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. Biol Reprod. **64**(6), 1761-1768.

Salzano, A., Albero, G., Zullo, G., Neglia, G., Abdel-Wahab, A., Bifulco, G., Zicarelli, L., Gasparrini, B. (2014). Effect of resveratrol supplementation during culture on the quality and cryotolerance of bovine in vitro produced embryos. Anim Reprod Sci. **151**(3-4), 91-96.

Sampaio, R.V., Conceição, S., Miranda, M.S., Sampaio, L. de F., Ohashi, O.M. (2012). MT3 melatonin binding site, MT1 and MT2 melatonin receptors are present in oocyte, but only MT1 is present in bovine blastocyst produced in vitro. Reprod Biol Endocrinol. **10**, 103.

Sawada, H., Yamazaki, K., Hoshi, M. (1990). Trypsin-like hatching protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. J Exp Zool. **254**(1), 83-87.

Schafer, F.Q., Buettner, G.R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. **30**(11), 1191-1212.

Sellem, E., Broekhuijse, M.L., Chevrier, L., Camugli, S., Schmitt, E., Schibler, L., Koenen, E.P. (2015). Use of combinations of in vitro quality assessments to predict fertility of bovine semen. Theriogenology. **84**(9), 1447-1454.

Sheth, B., Nowak, R.L., Anderson, R., Kwong, W.Y., Papenbrock, T., Fleming, T.P. (2008). Tight junction protein ZO-2 expression and relative function of ZO-1 and ZO-2 during mouse blastocyst formation. Exp Cell Res. **314**(18), 3356-3368.

Shi, D., Xu, J., Zhang, M., Niu, W., Shi, H., Yao, G., Li, Y., Zhang, N., Sun, Y. (2020). Association between the quality of inner cell mass and first trimester miscarriage after single blastocyst transfer. Reprod Biol Endocrinol. **18**(1), 43.

Simmet, K., Zakhartchenko, V., Philippou-Massier, J., Blum, H., Klymiuk, N., Wolf, E. (2018). OCT4/POU5F1 is required for NANOG expression in bovine blastocysts. Proc Natl Acad Sci U S A. **115**(11), 2770-2775.

Slominski, R.M., Reiter, R.J., Schlabritz-Loutsevitch, N., Ostrom, R.S., Slominski, A.T. (2012). Melatonin membrane receptors in peripheral tissues: distribution and functions. Mol Cell Endocrinol. **351**(2), 152-166.

Soto-Heras, S., Catalá, M.G., Roura, M., Menéndez-Blanco, I., Piras, A.R., Izquierdo, D., Paramio, M.T. (2019). Effects of melatonin on oocyte developmental competence and the role of melatonin receptor 1 in juvenile goats. Reprod Domest Anim. **54**(2), 381-390.

Soto-Heras, S., Paramio, M.T. (2020). Impact of oxidative stress on oocyte competence for in vitro embryo production programs. Res Vet Sci. **132**, 342-350.

Soto-Heras, S., Roura, M., Catalá, M.G., Menéndez-Blanco, I., Izquierdo, D., Fouladi-Nashta, A.A., Paramio, M.T. (2018). Beneficial effects of melatonin on in vitro embryo production from juvenile goat oocytes. Reprod Fertil Dev. **30**(2), 253-261.

Sovernigo, T.C., Adona, P.R., Monzani, P.S., Guemra, S., Barros, F., Lopes, F.G., Leal, C. (2017). Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. Reprod Domest Anim. **52**(4), 561-569.

Sponchiado, M., Gomes, N.S., Fontes, P.K., Martins, T., Del Collado, M., Pastore, A.A., Pugliesi, G., Nogueira, M.F.G., Binelli, M. (2017). Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PLoS One. **12**(4), e0175954.

Srivastava, N., Pande, M. (2016). Mitochondrion: Features, functions and comparative analysis of specific probes in detecting sperm cell damages. Asian Pacific Journal of Reproduction. **5**(6), 445-452.

Steeves, T.E., Gardner, D.K. (1999). Metabolism of glucose, pyruvate, and glutamine during the maturation of oocytes derived from pre-pubertal and adult cows. Mol Reprod Dev. **54**(1), 92-101.

Su, J., Wang, Y., Xing, X., Zhang, L., Sun, H., Zhang, Y. (2015). Melatonin significantly improves the developmental competence of bovine somatic cell nuclear transfer embryos. J Pineal Res. **59**(4), 455-468.

Tamura, H., Nakamura, Y., Korkmaz, A., Manchester, L.C., Tan, D.X., Sugino, N., Reiter, R.J. (2009). Melatonin and the ovary: physiological and pathophysiological implications. Fertil Steril. **92**(1), 328-343.

Tamura, H., Takasaki, A., Taketani, T., Tanabe, M., Kizuka, F., Lee, L., Tamura, I., Maekawa, R., Asada, H., Yamagata, Y., Sugino, N. (2013). Melatonin as a free radical scavenger in the ovarian follicle. Endocr J. **60**(1), 1-13.

Tamura, H., Takasaki, A., Taketani, T., Tanabe, M., Lee, L., Tamura, I., Maekawa, R., Aasada, H., Yamagata, Y., Sugino, N. (2014). Melatonin and female reproduction. J Obstet Gynaecol Res. **40**(1), 1-11.

Tan, D.X., Manchester, L.C., Liu, X., Rosales-Corral, S.A., Acuna-Castroviejo, D., Reiter, R.J. (2013). Mitochondria and chloroplasts as the original sites of melatonin synthesis: a hypothesis related to melatonin's primary function and evolution in eukaryotes. J Pineal Res. **54**(2), 127-138.

Tan, D.X., Manchester, L.C., Qin, L., Reiter, R.J. (2016). Melatonin: A Mitochondrial Targeting Molecule Involving Mitochondrial Protection and Dynamics. Int J Mol Sci. **17**(12), 2124.

Tan, D.X., Zheng, X., Kong, J., Manchester, L.C., Hardeland, R., Kim, S.J., Xu, X., Reiter, R.J. (2014) Fundamental issues related to the origin of melatonin and melatonin isomers during evolution: relation to their biological functions. Int J Mol Sci. **15**(9), 15858-90.

Tanabe, M., Tamura, H., Taketani, T., Okada, M., Lee, L., Tamura, I., Maekawa, R., Asada, H., Yamagata, Y., Sugino, N. (2015). Melatonin protects the integrity of granulosa cells by reducing oxidative stress in nuclei, mitochondria, and plasma membranes in mice. J Reprod Dev. **61**(1), 35-41.

Tang, X.L., Yang, X.Y., Jung, H.J., Kim, S.Y., Jung, S.Y., Choi, D.Y., Park, W.C., Park, H. (2009). Asiatic acid induces colon cancer cell growth inhibition and apoptosis through mitochondrial death cascade. Biol Pharm Bull. **32**(8), 1399-1405.

Tervit, H.R., Whittingham, D.G., Rowson, L.E. (1972). Successful culture in vitro of sheep and cattle ova. J Reprod Fertil. **30**(3), 493-497.

Tesfaye, D., Lonergan, P., Hoelker, M., Rings, F., Nganvongpanit, K., Havlicek, V., Besenfelder, U., Jennen, D., Tholen, E., Schellander, K. (2007). Suppression of connexin 43 and E-cadherin transcripts in in vitro derived bovine embryos following culture in vitro or in vivo in the homologous bovine oviduct. Mol Reprod Dev. **74**(8), 978-988.

Thomas, M., Jain, S., Kumar, G.P., Laloraya, M. (1997). A programmed oxyradical burst causes hatching of mouse blastocysts. J Cell Sci. **110** (Pt 14):1597-1602.

Tian, X., Wang, F., He, C., Zhang, L., Tan, D., Reiter, R.J., Xu, J., Ji, P., Liu, G. (2014). Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. J Pineal Res. **57**(3), 239-247.

Tian, X., Wang, F., Zhang, L., He, C., Ji, P., Wang, J., Zhang, Z., Lv, D., Abulizi, W., Wang, X., Lian, Z., Liu, G. (2017). Beneficial Effects of Melatonin on the In Vitro Maturation of Sheep Oocytes and Its Relation to Melatonin Receptors. Int J Mol Sci. **18**(4), 834.

Tijmes, M., Pedraza, R., Valladares, L. (1996). Melatonin in the rat testis: evidence for local synthesis. Steroids. 61(2), 65-68.

Tong, J., Sheng, S., Sun, Y., Li, H., Li, W.P., Zhang, C., Chen, Z.J. (2017). Melatonin levels in follicular fluid as markers for IVF outcomes and predicting ovarian reserve. Reproduction. **153**(4), 443-451.

Uyar, A., Seli, E. (2014). Metabolomic assessment of embryo viability. Semin Reprod Med. **32**(2), 141-152.

Van Soom, A., Ysebaert, M.T., de Kruif, A. (1997). Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. Mol Reprod Dev. **47**(1), 47-56.

Violette, M.I., Madan, P., Watson, A.J. (2006). Na+/K+ -ATPase regulates tight junction formation and function during mouse preimplantation development. Dev Biol. **289**(2), 406-419.

Vogel, M.W. (2002). Cell death, Bcl-2, Bax, and the cerebellum. Cerebellum. **1**(4), 277-287.

Wang, F., Tian, X., Zhang, L., Gao, C., He, C., Fu, Y., Ji, P., Li, Y., Li, N., Liu, G. (2014). Beneficial effects of melatonin on in vitro bovine embryonic development are mediated by melatonin receptor 1. J Pineal Res. **56**(3), 333-342.

Wang, T., Gao, Y.Y., Chen, L., Nie, Z.W., Cheng, W., Liu, X., Schatten, H., Zhang, X., Miao, Y.L. (2017). Melatonin prevents postovulatory oocyte aging and promotes subsequent embryonic development in the pig. Aging (Albany NY). **9**(6), 1552-1564.

Watson, A.J., Barcroft, L.C. (2001). Regulation of blastocyst formation. Front Biosci. **6**, D708-30.

Watson, A.J., Natale, D.R., Barcroft, L.C. (2004). Molecular regulation of blastocyst formation. Anim Reprod Sci. **82-83**, 583-592.

Wei, D., Zhang, C., Xie, J., Song, X., Yin, B., Liu, Q., Hu, L., Hao, H., Geng, J., Wang, P. (2013). Supplementation with low concentrations of melatonin improves nuclear maturation of human oocytes in vitro. J Assist Reprod Genet. **30**(7), 933-938.

Wolfenson, D., Roth, Z., Meidan, R. (2000). Impaired reproduction in heat-stressed cattle: basic and applied aspects. Anim Reprod Sci. **60-61**, 535-547.

Wongsrikeao, P., Nagai, T., Agung, B., Taniguchi, M., Kunishi, M., Suto, S., Otoi, T. (2007). Improvement of transgenic cloning efficiencies by culturing recipient oocytes and donor cells with antioxidant vitamins in cattle. Mol Reprod Dev. **74**(6), 694-702.

Woo, M.M., Tai, C.J., Kang, S.K., Nathwani, P.S., Pang, S.F., Leung, P.C. (2001). Direct action of melatonin in human granulosa-luteal cells. J Clin Endocrinol Metab. **86**(10), 4789-4797.

Wrenzycki, C., Herrmann, D., Niemann, H. (2007). Messenger RNA in oocytes and embryos in relation to embryo viability. Theriogenology. **68 Suppl 1**, S77-S83.

Xiao, L., Hu, J., Song, L., Zhang, Y., Dong, W., Jiang, Y., Zhang, Q., Yuan, .L, Zhao, X. (2019). Profile of melatonin and its receptors and synthesizing enzymes in cumulus-oocyte complexes of the developing sheep antral follicle-a potential estradiol-mediated mechanism. Reprod Biol Endocrinol. **17**(1):1.

Xie, Z., Chen, F., Li, W.A., Geng, X., Li, C., Meng, X., Feng, Y., Liu, W., Yu, F. (2017). A review of sleep disorders and melatonin. Neurol Res. **39**(6), 559-565.

Yang, L., Wang, Q., Cui, M., Li, Q., Mu, S., Zhao, Z. (2020). Effect of Melatonin on the In Vitro Maturation of Porcine Oocytes, Development of Parthenogenetically Activated Embryos, and Expression of Genes Related to the Oocyte Developmental Capability. Animals (Basel). **10**(2), 209.

Yang, M., Tao, J., Chai, M., Wu, H., Wang, J., Li, G., He., C., Xie, L., Ji, P., Dai, Y., Yang, L., Liu, G. (2017). Melatonin Improves the Quality of Inferior Bovine Oocytes and Promoted Their Subsequent IVF Embryo Development: Mechanisms and Results. Molecules. **22**(12), 2059.

Yang, M.Y., Rajamahendran, R. (2002). Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced in vitro. Anim Reprod Sci. **70**(3-4), 159-169.

Yang, W.C., Tang, K.Q., Fu, C.Z., Riaz, H., Zhang, Q., Zan, L.S. (2014). Melatonin regulates the development and function of bovine Sertoli cells via its receptors MT1 and MT2. Anim Reprod Sci. **147**(1-2), 10-16.

Yie, S.M., Niles, L.P., Younglai, E.V. (1995). Melatonin receptors on human granulosa cell membranes. J Clin Endocrinol Metab. **80**(5), 1747-1749.

Zaraza, J., Oropeza, A., Velazquez, M.A., Korsawe, K., Herrmann, D., Carnwath, J.W., Niemann, H. (2010). Developmental competence and mRNA expression of preimplantation in vitro-produced embryos from prepubertal and postpubertal cattle and their relationship with apoptosis after intraovarian administration of IGF-1. Theriogenology. **74**(1), 75-89.

Zavareh, S., Talebi, A., Hasanzadeh, H. (2015). Amending in vitro culture condition to overcome oxidative stress in assisted reproduction techniques (ART) J Paramed Sci. **6**(2), 135-148.

Zhang, H.M., Zhang, Y. (2014). Melatonin: a well-documented antioxidant with conditional pro-oxidant actions. J Pineal Res. **57**(2), 131-46.

Zhang, J., Yan, X., Tian, Y., Li, W., Wang, H., Li, Q., Li, Y., Li, Z., Wu, T. (2020). Synthesis of a New Water-Soluble Melatonin Derivative with Low Toxicity and a Strong Effect on Sleep Aid. ACS Omega. **5**(12), 6494-6499.

Zhang, M., Lin, J., Wang, S., Cheng, Z., Hu, J., Wang, T., Man, W., Yin, T., Guo, W., Gao, E., Reiter, R.J., Wang, H., Sun, D. (2017). Melatonin protects against diabetic cardiomyopathy through Mst1/Sirt3 signaling. J Pineal Res. **63**(2). e12418.

Zhang, X.Y., Xiong, Y.M., Tan, Y.J., Wang, .L, Li, R., Zhang, Y., Liu, X.M., Lin, X.H., Jin, L., Hu, Y.T., Tang, Z.H., Wu, Z.M., Yin, F.H., Wang, Z.Q., Xiao, Y., Sheng, J.Z., Huang, H.F. (2019). Melatonin rescues impaired penetration ability of human spermatozoa induced by mitochondrial dysfunction. Reproduction. **158**(5), 465-475.

Zhao, D., Yu, Y., Shen, Y., Liu, Q., Zhao, Z., Sharma, R., Reiter, R.J. (2019). Melatonin Synthesis and Function: Evolutionary History in Animals and Plants. Front Endocrinol (Lausanne). **10**, 249.

Zhao, X.M., Min, J.T., Du, W.H., Hao, H.S., Liu, Y., Qin, T., Wang, D., Zhu, H.B. (2015). Melatonin enhances the in vitro maturation and developmental potential of bovine oocytes denuded of the cumulus oophorus. Zygote. **23**(4), 525-536.

Zhao, Z., Yang, L., Zhang, D., Zheng, Z., Li, N., Li, Q., Cui, M. (2020). Elevation of MPF and MAPK gene expression, GSH content and mitochondrial distribution quality induced by melatonin promotes porcine oocyte maturation and development in vitro. PeerJ. **8**, e9913.

Zhu, Z., Li, R., Lv, Y., Zeng, W. (2019). Melatonin protects rabbit spermatozoa from cryo-damage via decreasing oxidative stress. Cryobiology. **88**, 1-8.

Zisapel, N. (2018). New perspectives on the role of melatonin in human sleep, circadian rhythms and their regulation. Br J Pharmacol. **175**(16), 3190-3199.

Zou, H., Chen, B., Ding, D., Gao, M., Chen, D., Liu, Y., Hao, Y., Zou, W., Ji, D., Zhou, P., Wei, Z., Cao, Y., Zhang, Z. (2020). Melatonin promotes the development of immature oocytes from the COH cycle into healthy offspring by protecting mitochondrial function. J Pineal Res. **68**(1), e12621.

7. Appendix

7.1 Ultrasound-guided follicular aspiration (OPU) control form

Institute
Department/IVF Lab
OPU Form

Farm:_____ **Location:**_____ **Barn:**_____
N° Process:_____ **Date:**_____ **Start time:**_____ **End time:**_____
OPU Technician: _____ **Lab Technician**_____

N°	Donor	Ct	Breed	Last OPU	Left Ovary (LO)				Right Ovary (RO)				COCs								
					D	VF	AF	OE	D	VF	AF	OE	G I	G II	G III	G IV	Dn	Ex	Dg	Total	MIV
1																					
2																					
3																					
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Caption

Category: (Ct): Cow: (C), Heifer: (H), Prepubertal (P). **Degree of difficulty to access the Ovary:** (D): **I:** Easy to manipulate, **II:** slightly difficult, **III:** Moderately difficult, **IV:** impossible to manipulate	**Visualized Follicles:** (VF): Number of follicles observed in the monitor.	**Aspirated Follicles:** (AF): Numbers of aspirated follicles.	**Others Structures:** (OE): Corpus luteum (CL), Cyst: (Follicular or Luteinized), others.
Cumulus Oocyte Complexes: (COCs): Quality grade: **I**: Excellent, **II**: Good, **III**: Regular, **IV**: Poor, **(Dn):** Denuded, **(Ex):** Expanded, **(Dg)**: Degenerated.			

Summary

Arrive to location:	____:____	**Total COCs:**		**Shipment**	
Start OPU:	____:____	**Total MIV:**		**Equipment:**	
Final OPU:	____:____	**% Viability**	_____%	**Temperature: °C**	_____°C
Departure:	____:____			**Time to arrival at the lab:**	____:____

Observations:

OPU Technician	By the Farm/Stable	Lab Technician
Name:	**Name:**	**Name:**

7.2 *In vitro* embryo production (IVP) control form

Institute **Form IVP N° _____**
Department/IVF Lab
IVP Control Form

Project: _____ **N° Process** _____ **Date:** _____
Lab Technician: _____ **Location:** _____

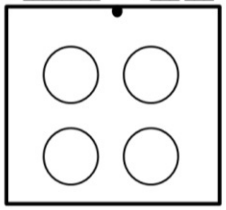
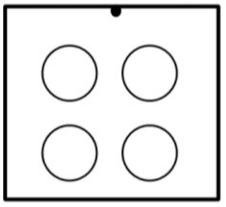
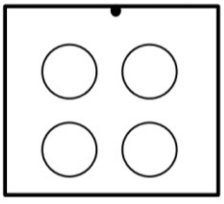
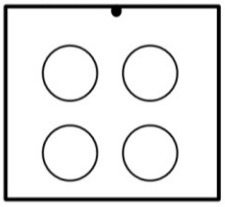
IVM information

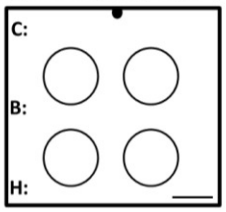
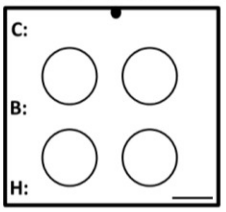
COCs retrieve method:	Shipment			Dishes setting:
Slicing:___ N° ovaries:_____ OPU:___ N° Cows:_____	Equipment:_____			Mediums:
Start OPU/Slicing:____:____ Final OPU/Slicing:___:___	Temperature:_____°C			IVM:_____
Total COCs:____ Total COCs MIV:____ % Viability:____	Time of arrival:_____/_____			IVF:_____
	Transport solution:_____			IVC:_____
Quality: GI:_____ GII:_____ GIII_____	Mixed of Gases (%)			Volume of medium:_____ µl
	CO_2	N_2	O_2	Volume of oil:_____ µl
Cumulus Oocyte Complexes: (COCs): Quality grade: I: Excellent, II: Good, III: Regular				N° of zygotes per wells:_____

IVF information

Semen Information
Bull:_____ Straw ID:_____Batch of semen:_____ Company:_____
Thawed:_____°C [spz] 1:_____ Motility 1:_____ Vitality: _____Morphology: _____
Spz separation method: _____Motility 2:_____ [spz] 2 :_____sperm/µl Vitality: ____Inseminating dose: ____µl /Drop

Dishes distribution

IVM	IVM	IVF	IVF
Dish: _____	Dish: _____	Dish: _____	Dish: _____
Treatment: _____	Treatment: _____	Treatment: _____	Treatment: _____
Date:_____Time: ___:___	Date:_____Time: ___:___	Date:_____Time: ___:___	Date:_____Time: ___:___
			
1:_____ 2:_____	1:_____ 2:_____	1:_____ 2:_____	1:_____ 2:_____
3:_____ 4:_____	3:_____ 4:_____	3:_____ 4:_____	3:_____ 4:_____
Exp. Rate: ___/____%	Exp. Rate: ___/____%	Hours post maturation:___	Hours post maturation:___

IVC	IVC	Embryo development	Embryo development
Dish: _____	Dish: _____	Dish: _____	Dish: _____
Treatment: _____	Treatment: _____	Treatment: _____	Treatment: _____
Date:_____Time: ___:___	Date:_____Time: ___:___	Cleavage: Day:_____	Cleavage: Day:_____
		Date:_____Time: ___:___	Date:_____Time: ___:___
1:_____ 2:_____	1:_____ 2:_____	1:_____% 2:_____%	1:_____% 2:_____%
3:_____ 4:_____	3:_____ 4:_____	3:_____% 4:_____%	3:_____% 4:_____%
Hours post insemination:___	Hours post insemination:___	Total:____/____:_____%	Total:____/____:_____%
		Blastocyst: Day:_____	Blastocyst: Day:_____
		Date:_____Time: ___:___	Date:_____Time: ___:___
		1:_____% 2:_____%	1:_____% 2:_____%
		3:_____% 4:_____%	3:_____% 4:_____%
		Total:____/____:_____%	Total:____/____:_____%

Observations:
_____
_____

7.3 *In vitro* embryo production (IVP) media composition

7.3.1 Medium for ovaries transport

Substance	Amount (g/ 1 L bidest H_2O)	Amount (g/ 5 L bidest H_2O)
NaCl	9	45
Penicillin G	0.06	0.3
Streptomycin sulphate	0.131	0.655

Stored at 4 °C for one week

7.3.2 Medium for oocyte recovery

7.3.2.1 Stock solution (100x)

Substance	Amount (500 ml)	Amount (1000 ml)
Na-Pyruvate	1.8 g	3.6 g
Streptomycin sulphate	2.37 g	4.74 g
D-Glucose x H_2O	54.99 g	100.98 g
$CaCl_2$	6.65 g	13.3 g
Penicillin G	3 g	6 g

Stored at -20 °C for 3 months

7.3.2.2 PBS solution

Substance	Manufacturer	Amount (g/ 1 L bidest H_2O)	Amount (g/ 5 L bidest H_2O)
PBS (Powder)	Applichem A0964	9.55 g	47.75 g
Stock solution (100x)		10 ml	50 ml

Stored at 4 °C for one week

7.3.3 Medium for OPU

Substance	Manufacturer	Amount
PBS solution		500 ml
Heparin sodium salt*	Applichem A30004,0005	25 mg
BSA*	Applichem A1391,0250	0.5 g

*Heparin and BSA were added on the day of the OPU session. Stored at 4 °C for one week

7.3.4 Medium for IVM

7.3.4.1 Holding medium outside of the incubator

Substance	Manufacturer	Amount (50 ml)	Amount (100 ml)
TCM199	Sigma M2520	0.735 g	1.47 g
Gentamycin sulphate	Sigma G3682	0.0025 g	0.005 g
Na-pyruvate	Applichem A3912	0.0011 g	0.0022 g
$NaHCO_3$	Sigma S4019	0.0175 g	0.035 g
BSA fatty acid-free	Sigma A7030	0.05 g	0.1 g
H_2O MilliQ		50 ml	100 ml

Stored at 4 °C for two weeks

7.3.4.2 Maturation medium

Substance	Manufacturer	Amount (50 ml)	Amount (100 ml)
TCM199	Sigma M2520	0.735 g	1.47 g
Gentamycin sulphate	Sigma G3682	0.0025 g	0.005 g
Na-pyruvate	Applichem A3912	0.0011 g	0.0022 g
$NaHCO_3$	Sigma S4019	0.11 g	0.22 g
H_2O MilliQ		To complete 50 g	To complete 100 g
BSA fatty acid-free	Sigma A7030	0.05 g	0.1 g
Hormones: eCG+hCG (Suigonan®)*	Intervet, Gmbh	10 i.u. eCG/ml and 5 i.u. hCG/ml	

*Hormones were added on the day of the *in vitro* maturation. Stored at 4 °C for two weeks

7.3.5 Medium for IVF (Fert-TALP: Tyrode's Albumin Lactate Pyruvate)

7.3.5.1 Stock solution

Substance	Manufacturer	Concentration (mM)	Amount (250 ml)
NaCl	Sigma S5886	114	1.6645 g
KCl	Sigma P5405	3.2	0.06 g
$NaHCO_3$	Sigma S4019	25	0.525 g
NaH_2PO_4 x H_2O	Merck 6346	0.3	0.0103 g
$CaCl_2$ x $2H_2O$	Sigma 21097	2	0.0735 g
$MgCl_2$	Ega-Chemie 20,833-7	0.5	0.012 g
Phenolred	Sigma P5530	0.01 µg/ml	0.0025 g
Penicillamine	Sigma P4875	20	0.0007 g
Na-lactate (60%)	Sigma L4263	10	0.465 g
H_2O MilliQ			250 ml

Stored at 4 °C for 3 months

7.3.5.2 APG (Albumin-Pyruvate-Gentamycin supplement)

- 1.2 g BSA fatty acid-free (Sigma A8806) in 20 ml Fert-TALP stock solution
- 10 mg Gentamycin (Sigma G3682) in 200 µl H_2O (MilliQ)
- 20 mg Na-Pyruvate (Applichem A3912) in 1 ml Fert-TALP stock solution
- Gentamycin solution transferred to Fert-TALP+BSA
- 140 µl of Na-Pyruvate solution transferred to Fert-TALP/BSA/Gentamycin solution

*Stored at -20 °C for 3 months

7.3.5.3 HHE (Hypotaurine-Heparin-Epinephrine supplement)

- Hypotaurine [1 mM/ (Sigma H1384)]
  - 1.09 mg Hypotaurine in 10 ml H_2O (MilliQ)
- Heparine [50 IU/ml (Applichem A30009)]
  - 2.6 mg Heparin in 10 ml H_2O (MilliQ)
- Epinephrine solution [250 µM (Sigma E4250)]

Substance	Manufacturer	Amount
Na-Lactate (60%)	Sigma L4263	165 mg
Na-metabisulfite	Riedel-De Haën AG	50 mg
H_2O MilliQ		50 ml
Epinephrine	Sigma E4250	2.3 mg

7.3.5.4 HHE (Hypotaurine-Heparin-Epinephrine supplement) working solution

Substance	Amount
Hypotaurine solution	10 ml
Epinephrine solution	4 ml
H_2O MilliQ	26 ml
Filter	
Hypotaurine + Epinephrine solutions	8 ml
Heparine solution	4 ml

Stored at -20 °C for 3 months

7.3.6 Medium for IVC (Synthetic Oviductal Fluid: SOFaa culture medium)

7.3.6.1 SOF-Stock A (10x)

Substance	Manufacturer	Concentration In stock	Amount (50 ml)
NaCl	Sigma S5886	1.08 M	3.145 g
KCl	Sigma P5405	0.072 M	0.267 g
KH_2PO_4	Sigma P5655	0.012 M	0.081 g
$MgSO_4$	Sigma 2643	0.015 M	0.091 g
H_2O MilliQ			49.2 ml
Na-Lactate	Sigma L4263	0.042	0.3 ml

Filtered and stored at 4 °C for 4 weeks

7.3.6.2 SOF-Stock B (10x)

Substance	Manufacturer	Concentration In stock	Amount (50 ml)
$NaHCO_3$	Sigma S4019	0.25 M	1.050 g
Phenolred	Sigma P5530		0.005 g
H_2O MilliQ			50 ml

Filtered and stored at 4 °C for 4 weeks

7.3.6.3 SOF-Stock C (100x)

Substance	Manufacturer	Concentration In stock	Amount (5 ml)
Na-Pyruvate	Sigma P3662	0.073 M	0.04 g
H_2O MilliQ			5 ml

Filtered and stored at 4 °C for 4 weeks

7.3.6.4 SOF-Stock D (100x)

Substance	Manufacturer	Concentration In stock	Amount (5 ml)
$CaCl_2$ x $2H_2O$	Sigma C7902	0.178 M	0.131 g
H_2O MilliQ			5 ml

Filtered and stored at 4 °C for 4 weeks

7.3.6.5 Glutamine stock

Substance	Manufacturer	Amount (10 ml)
Glutamine	Sigma G6392	0.292 g
H_2O MilliQ		10 ml

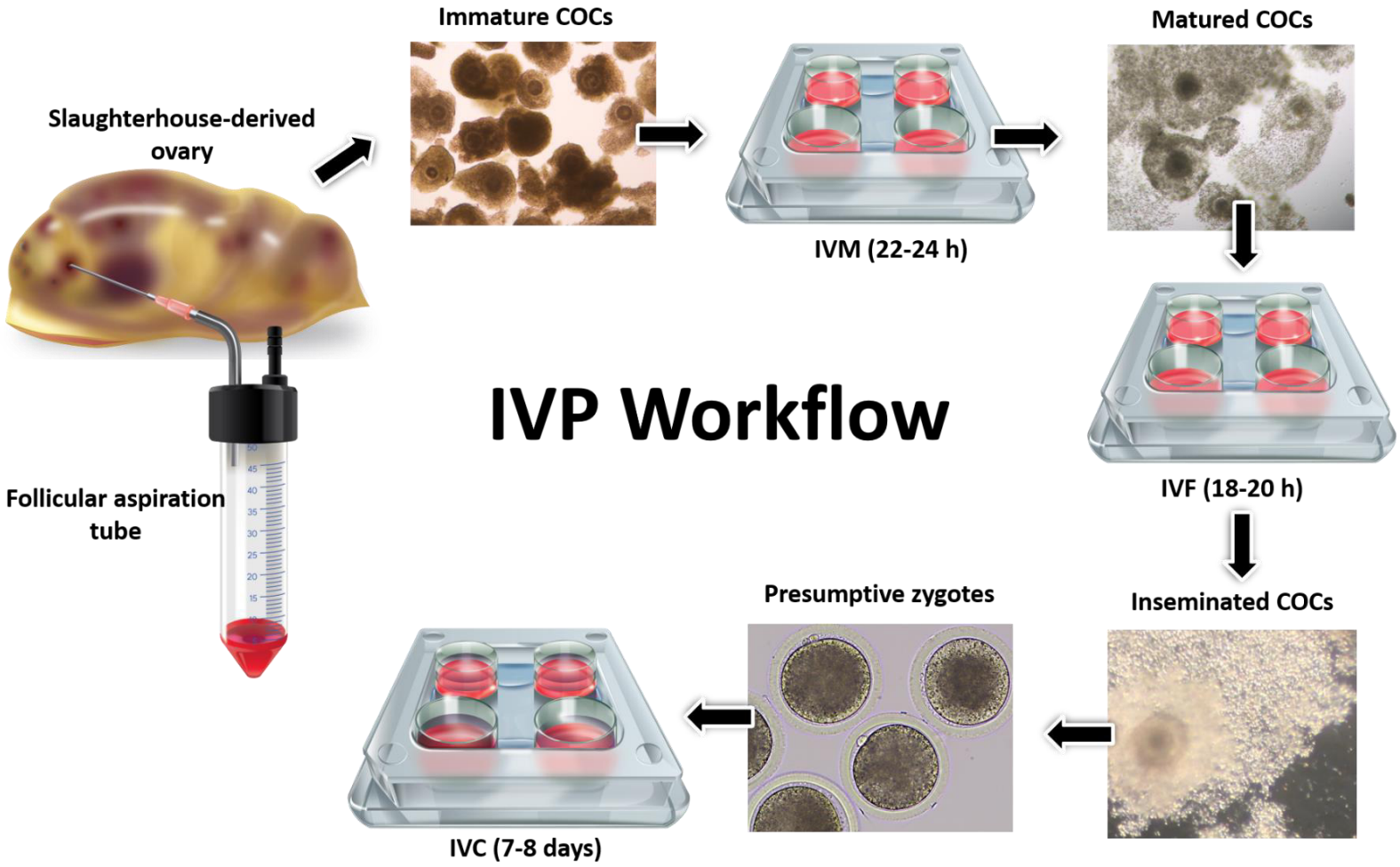
Stored at -20 °C for 3 months

7.3.6.6 SOFaa(m) IVC culture medium

Substance	Manufacturer	Concentration	Amount (50 ml)
myo-Inositol	Sigma I7508	2.77 mM	0.025 g
Gentamycin	Sigma G3632	50 µg/ml	0.0025 g
H_2O MilliQ			39 ml
Glutamine stock	Glutamine	0.2 mM	50 µl
Stock A (10x)	Salts		5 ml
Stock B (10x)	$NaHCO_3$		5 ml
Stock C (100x)	Na-Pyruvate		0.5 ml
Stock D (100x)	$CaCl_2$		0.5 ml
BME (50x)	Sigma B6766	30 µl/ml	1.5 ml
MEM (100x)	Sigma (M7145)	10 µl/ml	0.5 ml
BSA fatty acid-free	Sigma A7030		0.2 g
H_2O MilliQ			50 ml

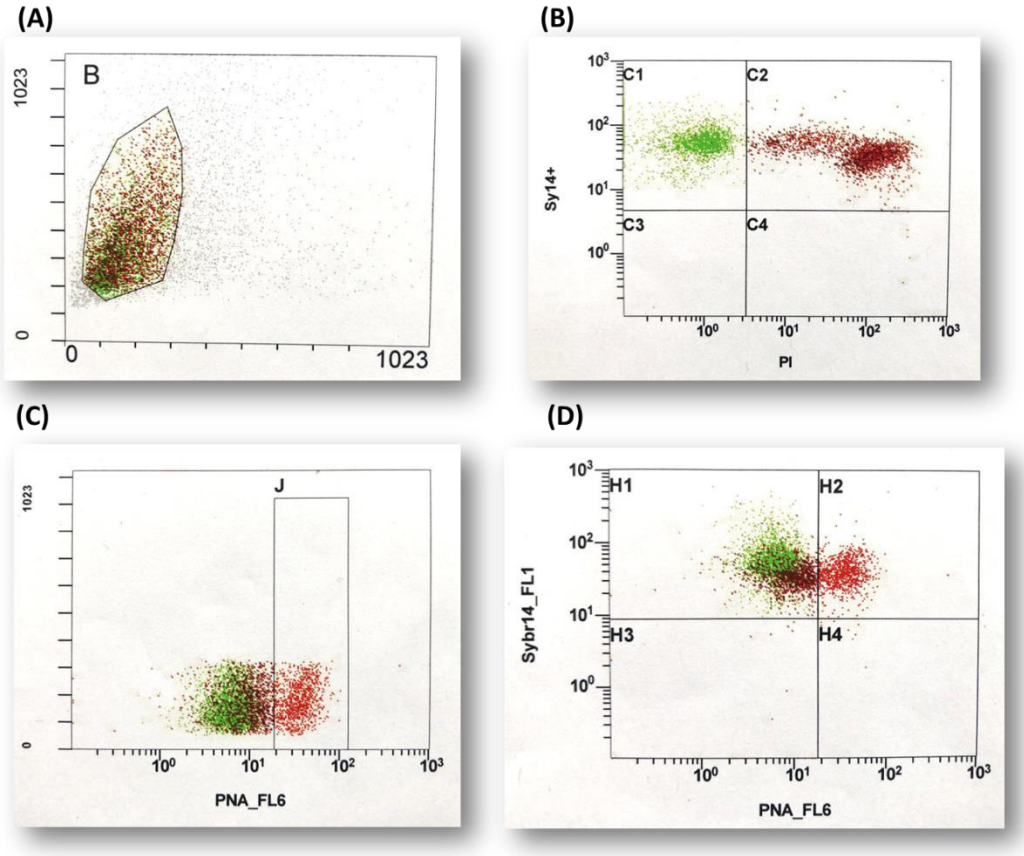
BME: Basal Medium Eagle (Amino Acids Solution 50× without L-glutamine). MEM: Minimum Essential Medium (Non-essential Amino Acid Solution 100x). Stored at 4 °C for 2 weeks

7.3.7 *In vitro* embryo production workflow



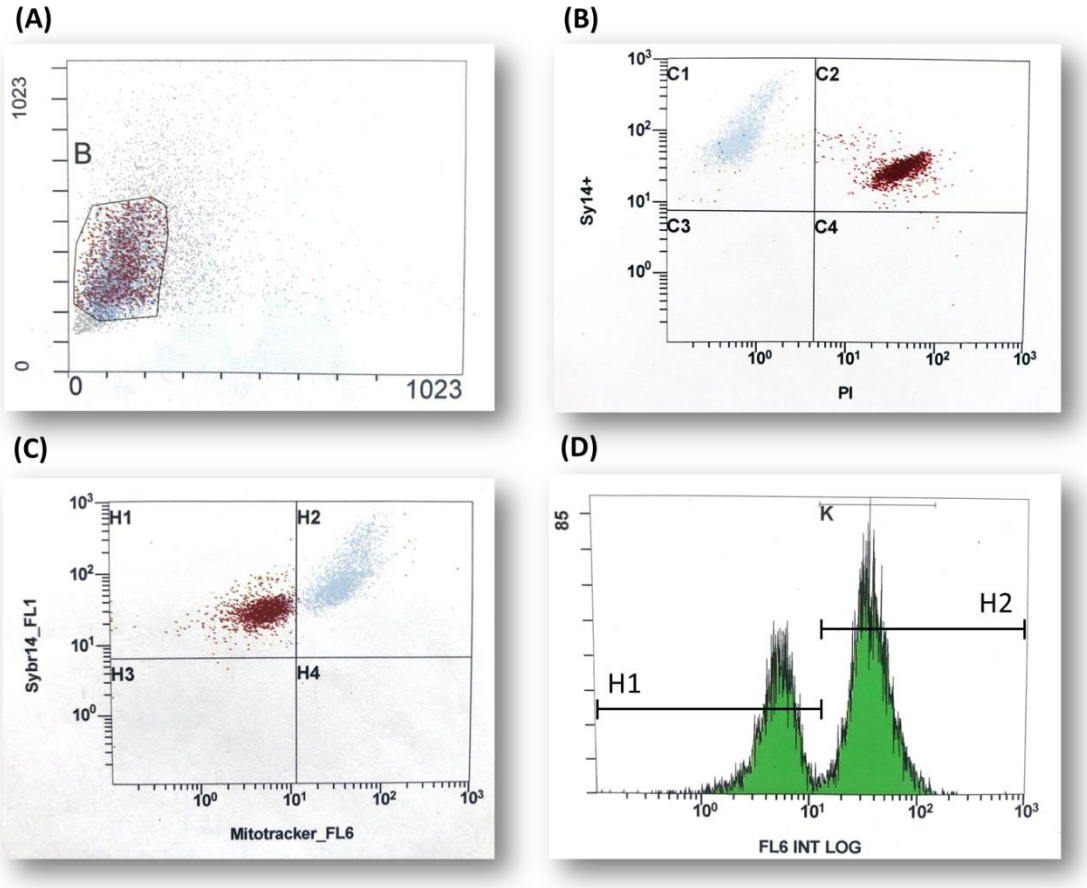
7.4 Flow cytometric evaluation of spermatozoa

7.4.1 Gating strategy for plasma membrane and acrosome integrity



Sybr14/PI/PNA-Alexa Fluor™ 647 (PNA-AF647). Example of the detection plots acquired with the Gallios cytometer and gating for plasma membrane and acrosome integrity. (A) Gating in the forward scatter/side scatters projection to separate intact sperm from other signals (debris and noise). (B) Gating in the FL1/FL3 projection determines the number of sperm with intact plasma membrane (Sybr14+/PI-; Quadrant C1). (C) Gating in the FL6 projection to determine Peanut agglutinin (PNA) (PNA-Alexa Fluor™ 647 (Sybr14 positive/PI negative/ PNA-AF647 negative). (D) Gating in the FL6 projection determines sperm with intact plasma membrane and acrosome integrity [Quadrant H1: Live/viable sperm/Intact acrosome, Quadrant H2: Dead sperm/Damage reacted acrosome].

7.4.2 Gating strategy for sperm viability and mitochondrial membrane potential in viable spermatozoa



Sybr14/PI/Mito Tracker™ Deep red FM (Deep Red FM). Example of the detection plots acquired with the Gallios cytometer and gating for sperm viability and mitochondrial membrane potential in viable spermatozoa. (A) Gating in the forward scatter/side scatters projection to separate sperm from other signals (debris and noise). (B) Gating in the FL1/FL3 projection determines the number of sperm with intact plasma membrane (Sybr14+/PI-; Quadrant C1). (C) Gating in the FL6 projection to determine mitochondria membrane potential in living sperm cells (Sybr14+/PI-) [Quadrant H1: Low mitochondrial membrane potential, Quadrant H2: High mitochondrial membrane potential]. (D) Histogram showing spermatozoa with high (Marker H2) and low (Marker H1) mitochondrial membrane potential.

Affidavit

I herewith affirm that I am the sole author of the thesis titled "Role of melatonin in bovine gametes competence and preimplantation embryo development *in vitro*", which was written according to the principles of good scientific practice. No third party assistance has been used, except the support mentioned under Acknowledgments.

I did not make use of any paid dissertation services or other consultants. Nor did anyone receive unpaid services from me for work related to the contents of the submitted thesis.

The thesis was completed at the following institutions:

Institute of Farm Animal Genetics – Friedrich-Loeffler-Institut (Mariensee)

This thesis has not been previously submitted for evaluation for admission to an examination, doctoral graduation, or any such purpose.

I herewith declare that I have been informed about the significance of this affidavit and have been made aware of the provisions of § 156 of the German criminal code (StGB).

_____

Date, Signature

Acknowledgments

I give thanks to you, O lord my God, with my whole heart, and I will glorify your name forever; **Psalm 86:12**

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