Universitat Autònoma de Barcelona

HEAT SHOCK PROTEINS: A STRATEGY TO IMPROVE BOVINE OOCYTE VITRIFICATION

Meritxell Vendrell i Flotats

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TESI DOCTORAL PRESENTADA PER:

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CERTIFIQUEN:

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I per a què consti als efectes oportuns, signen la present a Bellaterra, 6 de juny de 2019.

Dra Teresa Mogas Amorós Dr Manel López Béjar

La realització d'aquest treball ha estat possible gràcies al suport del *Ministerio de Ciencia, Innovación y Universidades del Gobierno de España* a través d'una beca predoctoral de Formació de Personal Investigador (BES-2014-071075), i al finançament proporcionat pel *Ministerio de Economía y Competitividad* (Projecte AGL2013–46769 i AGL2016-79802-P). Així com també d'una ajuda de l'administració pública per fer una estada a l'estranger (EEBB-I-17-12536), també parcialment finançada per l'OCDE.

"Estima el teu ofici, la teva vocació, la teva estrella, allò pel qual serveixes, allò en el qual et sents un entre els homes. Esforça't en la teva tasca com si de cada detall que penses, de cada mot que dius, de cada peça que hi poses, de cada cop del teu martell, en depengués la salvació de la humanitat. Car en depèn, creu-me. Si, oblidat de tu mateix, fas tot el que pots en el teu treball, fas més que un emperador que regís automàticament els seus estats; fas més que el qui inventa teories universals només per satisfer la seva vanitat, fas més que el polític, que l'agitador, que el qui governa. Pots negligir tot això i l'adobament del món. *El món s'arreglaria bé tot sol, només que tothom fes el seu deure amb amor* a casa seva".

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INDEX OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2c	2-cell embryo
8c	8-cell embryo
AKT	AKT serine/threonine kinase 1
ANOVA	Analysis of the variance
BAD	BCL2 associated agonist of cell death
BAX	BCL2 associated X, apoptosis regulator
BCL2L1	BCL2 like 1
BI	Blastocyst
BSA	Bovine serum albumin
cDNA	Complementary DNA
CNTF	Ciliary neurotrophic factor
COC	Cumulus-oocyte complex
CPA	Cryoprotectant
CT-1	Cardiotrophin 1
CLCF1	Cardiotrophin-like cytokine factor 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3 alpha
DPPA3	Developmental pluripotency associate 3
EG	Ethylene glycol
EGA	Embryonic genome activation
Exp	Expanded blastocyst
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GP130	Glycoprotein 130
GV	Germinal vesicle
Hd	Hatched blastocyst
HDAC1	Histone deacetylase 1
Hg	Hatching blastocyst
HS	Heat stress or heat shock
HSE	Heat shock element

HSF1	Heat shock factor 1
HSP	Heat shock protein
HSP70	Heat shock protein 70 kDa
HSP90	Heat shok protein 90 kDa
HSPA1A	Heat shock protein family A (Hsp70) member 1A
HSP90AA1	Heat shock protein 90 alpha family class A member 1
HSR	Heat shock response
H2AFZ	H2A histone family, member Z
KAT2A	Lysine acetyltransferase 2A
ICM	Inner cell mass
IκB	I kappa B kinases
IKK	IκB kinase
IL-11	Interleukin-11
IL-11R	Interleukin-11 receptor
IL-35	Interleukin-35
IL-39	Interleukin-39
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IM	Immature oocytes in germinal vesicle stage
IU	International unit
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
JAK	Jak family tyrosine kinases or Janus kinases
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
LPS	Lipopolysaccharide
MII	Mature oocytes or metaphase II oocytes
MEG	Maternal effect genes
MET	Maternal-to-embryonic transition
miRNA	microRNA
miR-146a	microRNA-146a
miR-155	microRNA-155
miR-21	microRNA-21
miR-34c	microRNA-34c
NF-kB	Nuclear factor kappa B

NPM2	Nucleoplasmin 2
NS	Non-supplemented
nt	Nucleotide
OSM	Oncostatin M
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (4,5)-diphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
pre-miRNA	Precursor-microRNA
pri-miRNA	Primary-microRNA
PVA	Polyvinyl alcohol
RNA	Ribonucleic acid
snRNA	small nuclear RNA
SOF	Synthetic oviductal fluid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
TAB2	TGF-beta activated kinase 1 (MAP3K7) binding protein 2
TCM-199	Tissue culture medium 199
***TGF*β**	Transforming growth factor beta 1
TLR4	Toll like receptor 4
ZAR1	Zygote arrest 1

SUMMARY

SUMMARY

Gamete cryopreservation, as many other reproductive technologies, has become a necessity for an efficient production of domestic animals, allowing the modification of animal breeding programs and offering a mean to maintain genetic diversity by banking important germplasm that could reinvigorate future populations. Achieving a robust cryopreservation method would guarantee the preservation of feminine genetic material of endangered species and of those of a great economic importance. Vitification is the most promising technique for oocyte cryopreservation. From the first attempts of vitrifiying bovine oocytes the vitrification protocol and the support devices used have changed considerably. However, due to the peculiarities of the bovine oocyte, fertilization rate and developmental competence of bovine cryopreserved oocytes still need to be improved. During the vitrification process cells suffer cryodamage and ice formation, what leads to ultrastructural changes driving to maturation or development impairment. For this purpose, in this thesis we have investigated different strategies to ameliorate the developmental competence of bovine oocytes after vitrification and warming processes.

Heat shock proteins (HSPs) are highly conserved cellular stress proteins that have been identified as a cell defense mechanism. Most of them are constitutively expressed at low levels but some can be upregulated in response to cellular stressors so to regulate protein folding, protect against protein aggregation and denaturation. Among the inducible ones, HSP70 *(HSPA1A)* and HSP90 *(HSP90AA1)* were those analyzed in this thesis. In chapter II, we can observe how a defined heat shock treatment (1h, 41.5ºC) increases fluorescence intensity for HSP70 when applied at 8h of IVM. When this heat stress was applied before oocyte vitification, it did not mitigate the harmful effects of cryopreservation how me hypothesized.

Follicular fluid is naturally rich in growth factors and cytokines. Interleukin (IL)-6 family of cytokines is known to have a predominant role in reproduction function. In chapter III, we assessed how IL-6, IL-11 and leukemia inhibitory factor (LIF) induced changes in the expression of miR-21 and other key miRNAs in bovine cumulus cells and oocyte. We did not find promising data for IL-6 and IL-

11, but LIF did enhance the expression of miR-21 in both cumulus cells and oocytes.

LIF has been described to activate key signaling pathways for cell survival and maintenance. In chapter IV, we evaluated how LIF supplementation affected embryo development and modulated gene expression in oocytes and embryos. LIF increased expression of *HSPA1A* and *HSP90AA1* at the time of embryonic genome activation. By the time embryos developed, at the expanded and hatched stage, *HSPA1A* was reduced and so did *DNMT3A*. LIF did not influence on cleavage and blastocyst rate. The greater impact exerted by LIF was during the maternal-to-embryonic transition.

Mimicking physiological conditions, and guided for the results obtained previously, on chapter V we supplemented oocytes with LIF during maturation in order to improve vitrification. Vitrification modifies clearly mRNA levels and LIF seemed to precondition oocytes not to suffer so much stress, although no differences were observed in any treatment group in terms of blastocyst rate.

RESUM

La crioconservació de gàmetes, igual que moltes altres tecnologies reproductives, s'ha convertit en una necessitat per a una producció eficient d'animals domèstics, permetent la modificació dels programes de cria d'animals i oferint un mitjà per a mantenir la diversitat genètica mitjançant l'emmagatzematge d'important germoplasma que podria revitalitzar les poblacions futures. Aconseguir un mètode robust de crioconservació garantiria la preservació del material genètic femení d'espècies en perill d'extinció i de gran importància econòmica. La vitificació és la tècnica més prometedora per a la crioconservació d'oòcits. Des dels primers intents de vitrificació d'oòcits bovins, el protocol de vitrificació i els dispositius de suport utilitzats han canviat considerablement. No obstant això, a causa de les peculiaritats de l'oòcit boví, la taxa de fecundació i la capacitat de desenvolupament dels oòcits crioconservats bovins encara han de millorar-se. Durant el procés de vitrificació, les cèl·lules sofreixen crio-dany i formació de gel, la qual cosa condueix a canvis ultraestructurals que provoquen fallades en la maduració i el desenvolupament. Per a aquest propòsit, en aquesta tesi hem investigat diferents estratègies per a millorar la capacitat de desenvolupament dels oòcits bovins després dels processos de vitrificació i escalfament.

Les proteïnes de xoc tèrmic (HSPs) són proteïnes d'estrès cel·lular altament conservades que s'han identificat com un mecanisme de defensa cel·lular. La majoria d'elles s'expressen de forma constitutiva a nivells baixos, però algunes es poden regular positivament en resposta a factors estressants cel·lulars per a controlar el plegament de proteïnes i protegir contra l'agregació i desnaturalització de proteïnes. Entre les proteïnes induïbles, en aquesta tesi van ser analitzades la HSP70 (HSPA1A) i la HSP90 (HSP90AA1). En el capítol II, podem observar com un tractament de xoc tèrmic definit (1h, 41.5 °C) va augmentar la intensitat de la fluorescència per a la HSP70 quan es va aplicar a les 8h d'IVM. Quan aquest estrès per calor es va aplicar abans de la vitificació dels oòcits, no es van mitigar els efectes nocius de la crioconservació, com prèviament hi havia hipotetitzat.

El líquid fol·licular és naturalment ric en factors de creixement i citocines. Se sap que la família de citocines interleuquina (IL) -6 té un paper predominant en la funció de reproducció. En el capítol III, avaluem com la IL-6, la IL-11 i el factor inhibidor de la leucèmia (LIF) induïen canvis en l'expressió de miR-21 i uns altres miRNAs claus en cèl·lules de cúmuls i oòcits bovins. No trobem dades prometedores per a IL-6 i IL-11, però LIF va augmentar l'expressió de miR-21tant en cèl·lules de cúmul com en oòcits.

S'ha descrit el paper del LIF en l'activació de vies de senyalització clau per a la supervivència i el manteniment de les cèl·lules. En el capítol IV, avaluem com la suplementació amb LIF afectava el desenvolupament d'embrions i modulava l'expressió gènica en oòcits i embrions. LIF va augmentar l'expressió d'HSPA1A i *HSP90AA1* en el moment de l'activació del genoma embrionari. En el moment en què es van desenvolupar els embrions, en l'etapa expandida i eclosionada, la *HSPA1A* es va reduir i també ho va fer el *DNMT3A*. LIF no va influir en la divisió i la taxa de blastocists. El major impacte exercit per LIF va ser durant la transició matern-embrionària.

Imitant les condicions fisiològiques i guiats pels resultats obtinguts anteriorment, en el capítol V suplementaren els oòcits amb LIF durant la maduració per a millorar la vitrificació. La vitrificació va modificar clarament els nivells de ARNm i el LIF sembla precondicionar als oòcits perquè no sofreixin tant estrès, encara que no es van observar diferències en cap grup de tractament en termes de taxa de blastocists.

RESUMEN

La crioconservación de gametos, al igual que muchas otras tecnologías reproductivas, se ha convertido en una necesidad para una producción eficiente de animales domésticos, permitiendo la modificación de los programas de cría de animales y ofreciendo un medio para mantener la diversidad genética mediante el almacenamiento de importante germoplasma que podría revitalizar las poblaciones futuras. Lograr un método robusto de crioconservación garantizaría la preservación del material genético femenino de especies en peligro de extinción y de gran importancia económica. La vitificación es la técnica más prometedora para la crioconservación de ovocitos. Desde los primeros intentos de vitrificación de ovocitos bovinos, el protocolo de vitrificación y los dispositivos de soporte utilizados han cambiado considerablemente. Sin embargo, debido a las peculiaridades del ovocito bovino, la tasa de fecundación y la capacidad de desarrollo de los ovocitos crioconservados bovinos aún deben mejorarse. Durante el proceso de vitrificación, las células sufren crio-daño y formación de hielo, lo que conduce a cambios ultraestructurales que provocan fallos en la maduración y el desarrollo. Para este propósito, en esta tesis hemos investigado diferentes estrategias para mejorar la capacidad de desarrollo de los ovocitos bovinos después de los procesos de vitrificación y calentamiento.

Las proteínas de choque térmico (HSPs) son proteínas de estrés celular altamente conservadas que se han identificado como un mecanismo de defensa celular. La mayoría de ellas se expresan de forma constitutiva a niveles bajos, pero algunas se pueden regular positivamente en respuesta a los factores estresantes celulares para controlar el plegamiento de proteínas y proteger contra la agregación y desnaturalización de proteínas. Entre las proteínas inducibles, en esta tesis fueron analizadas la HSP70 (*HSPA1A*) y la HSP90 (*HSP90AA1*). En el capítulo II, podemos observar cómo un tratamiento de choque térmico definido (1h, 41.5ºC) aumentó la intensidad de la fluorescencia para la HSP70 cuando se aplicó a las 8h de IVM. Cuando este estrés por calor se aplicó antes de la vitificación de los ovocitos, no se mitigaron los efectos nocivos de la crioconservación, como previamente había hipotetizado.

El líquido folicular es naturalmente rico en factores de crecimiento y citoquinas. Se sabe que la familia de citoquinas interleucina (IL) -6 tiene un papel predominante en la función de reproducción. En el capítulo III, evaluamos cómo la IL-6, la IL-11 y el factor inhibidor de la leucemia (LIF) inducían cambios en la expresión de miR-21 y otros miRNAs claves en células de cúmulos y ovocitos Bovinos. No encontramos datos prometedores para IL-6 e IL-11, pero LIF aumentó la expresión de miR-21 tanto en células de cúmulo como en ovocitos.

Se ha descrito el papel del LIF en la activación de vías de señalización claves para la supervivencia y el mantenimiento de las células. En el capítulo IV, evaluamos cómo la suplementación con LIF afectaba el desarrollo de embriones y modulaba la expresión génica en ovocitos y embriones. LIF aumentó la expresión de *HSPA1A* y *HSP90AA1* en el momento de la activación del genoma embrionario. En el momento en que se desarrollaron los embriones, en la etapa expandida y eclosionada, se redujo la *HSPA1A* así como el *DNMT3A*. LIF no influyó en la división y la tasa de blastocitos. El mayor impacto ejercido por LIF fue durante la transición materno-embrionaria.

Imitando las condiciones fisiológicas y guiados por los resultados obtenidos anteriormente, en el capítulo V suplementamos los ovocitos con LIF durante la maduración para mejorar la vitrificación. La vitrificación modificó claramente los niveles de ARNm y el LIF parece precondicionar a los ovocitos para que no sufran tanto estrés, aunque no se observaron diferencias en ningún grupo de tratamiento en términos de tasa de blastocitos.

OBJECTIVES

The objective of the present thesis was to induce the expression of the heat shock proteins in order to improve bovine oocyte vitrification and the subsequent developmental competence.

The specific objectives are as follows:

1. To evaluate the protective effect of exposure of bovine oocytes to moderate heat stress during in vitro maturation prior vitrification

2. To determine whether the IL-6 family members LIF (25 gn/mL), IL-6 (10 ng/mL) or IL-11 (5 ng/mL) induced changes in the expression of miR-21 and other key miRNAs in bovine cumulus cells and oocyte cultured *in vitro* as cumulus oocyte complexes

3. To evaluate the effect of LIF supplementation during bovine oocyte maturation on embryo development and gene expression

4. To characterize the effect of LIF supplementation during bovine oocyte maturation on the vitrification process. Embryo development and gene expression will be assessed in order to elucidate the cytokine impact

With the aim to reach these objectives, four different studies were performed. The first study was entitled *"Effect of heat stress during in vitro maturation on developmental competence of vitrified bovine oocytes"* (Chapter II) and influenced in the assessment of specific objectives 1. The second study, named "*Effects of IL-6 family members on specific miRNA expression during in vitro oocyte maturation"* (Chapter III), was designed for the specific objective 2. The third study was *"Effect of the addition of LIF during in vitro maturation on embryo development and gene expression"* (Chapter IV) and allowed to reach specific objective 3. Finally in order to optimize vitrification, *"Gene expression profiles and* in vitro *development following vitrification of bovine oocytes matured in medium supplemented with LIF"* was conducted to reach the specific objective 4.

Chapter I

General Introduction

1. THE CHALLENGE OF OOCYTE CRYOPRESERVATION

Gamete cryopreservation, as many other reproductive technologies, has become a necessity for an efficient production of domestic animals, allowing the modification of animal breeding programs and offering a mean to maintain genetic diversity by banking important germplasm that could reinvigorate future populations[1]. Achieving a robust cryopreservation method would guarantee the preservation of feminine genetic material of endangered species and of those of a great economic importance. And that is the big challenge, freezing a huge peculiar cell without generating crystals and getting a thawed fully competent oocyte. All the difficulties will be discussed later on.

The basis of cryopreservation are founded on Gay-Lussac's discovery in 1804 when, mounted in a hot air balloon, he realized small drops of water could be cooled to subzero temperatures being kept at a liquid state. Nowadays, 200 years later, two cryopreservation methods are in use: slow freezing and vitrification. Both techniques were developed to diminish freezing injuries. Slow freezing consists of the application of controlled cooling rates and cell dehydration caused by the cryoprotectants (CPAs). This method is characterized to use low CPAs concentrations, an advantage due to their known toxicity and osmotic shock that provided early results in mouse[2]–[4]. Even though, slow freezing has not been successful cryopreserving species more sensitive to chilling (pig, cattle, sheep and horse).

Vitrification is characterized by transforming the solution into an amorphous state and, instead, requires a high CPAs concentration, reason why it took more time to achieve positive results. From the first attempts of vitrifiying bovine oocytes in 1992[5], [6], the vitrification protocol and the support devices used have changed considerably, trying to improve the key factors for a successful vitrification (reviewed by[7], [8]):

a. *High cooling and warming rate:*

   To reduce the probability of ice formation, temperature should decrease drastically and it can only be achieved by plunging the sample into LN_2. On the other hand, the high warming rate is achieved with the immersion of the sample in a warm bath.

b. *High viscosity:*

   Vitrification solution has a high viscosity because of the presence of CPAs, permeable (DMSO and EG) and non-permeable (sucrose or trehalose). Increasing the CPAs concentration, increased the glass transition temperature, thus lowering the chance of ice nucleation and crystallization

c. *Volume:*

   The volume of the solution where the oocyte is vitrified should be minimized because small volumes allow better heat transfer. Among all the devices that have been developed, the Cryotop® method[9] seems to be reaching the smallest volumes, a minimum of 0.1 μL. Our group has obtained noteworthy viability rates with this device[10]–[14]. This is the system used in all the vitrification experiments of this thesis.

$$Probability\ of\ vitrification\ = \frac{cooling\ \&\ warming\ rate \times viscosity}{volume}$$

However, fertilization rate and developmental competence of bovine cryopreserved oocytes still need to be improved. The peculiarities of the bovine oocyte make it more difficult to accomplish a successful vitrification:

a. *Surface to volume ratio:* The oocyte is a large cell with a low surface to volume ratio, decreasing the cooling rate.
b. *Lipid droplets:* the cytoplasm presents a high content of lipid droplets[15] that increase chilling sensitivity.

c. *Cellular membrane:* Membranes rich in cholesterol are more fluid and flexible at lower temperatures and therefore less sensitive to cryodamage. The cholesterol/phospholipids ratio of the bovine oocyte membranes is low and the incorporation of cholesterol prior vitrificaton has also been studied[12], [16]. Water can move through membranes by simple diffusion or by facilitated diffusion across channels. Bovine oocyte membrane presents a low permeability coefficient partly due to a lack of aquaporin expression[17] making the oocyte more sensitive to cryopreservation.
d. *Cytoskeleton:* Mature oocyte presents the actin microfilaments arranged as a thin subcortical layer conferring integrity to the cell; and microtubules organized forming the meiotic spindle. Both actin microfilaments[18], [19] and microtubules disorganize and/or depolymerize with cryopreservation[10], [20].
e. *Cortical granules:* Cooling and ice formation can result in premature release of cortical granules leading to zona hardening[21]–[23] and impairing fertilization.
f. *Calcium:* Exposure to CPAs and low temperatures induce a rise of intracellular calcium causing DNA fragmentation[24]; the presence of calcium during cryopreservation would significantly affect the developmental competence of oocytes[25].
g. *Mitochondria:* vitrification causes abnormal mitochondrial appearance and distribution[10], [22], [26] .
h. *DNA:* Chromosomal dispersal and DNA abnormalities[10], [27], [28].

Cooling and ice formation can entail some of these detailed alterations, leading to low outcomes in fertilization and blastocyst rate. Hence, developing new strategies is necessary to reach an optimized method to preserve genetic material.

2. HEAT SHOCK PROTEINS

Heat Shock Proteins (HSPs) are highly conserved cellular stress proteins present both in prokaryotic and eukaryotic cells. The first description of a cellular heat stress response was observed in 1962 by Ritossa, where the salivary gland chromosomes of *Drosophila melanogaster* exhibited a characteristic puffing pattern after a transient heat exposure. HSPs have been identified as a defense mechanism for cell survival under severe conditions. Most of them are constitutively expressed at low levels but some can be up-regulated in response to cellular stressors (physiological, chemical, nutritional or environmental) so to regulate protein folding and to protect cellular proteins against aggregation and denaturation. Under normal physiological conditions, HSPs also participate in a number of normal cellular functions, including metabolism, growth, differentiation and apoptosis, where they mainly assure adequate folding, transport and complex assembly of newly synthesized proteins[29].

HSPs are a huge family of molecular chaperones that differ basically in their structure and molecular weight. Over the years, the nomenclature and classification has varied according to the advances, being hard sometimes to find a consensus (Table I-1). However, mammalian HSPs have been traditionally classified according to their molecular size: small HSPs (15 to 30kDa), DNAJ (HSP40), chaperonins (HSP60), HSP70 and HSP90. High molecular weight HSPs are ATP-dependent chaperones and require co-chaperones to modulate their conformation and ATP binding (reviewed by [30], [31]. All families of HSPs contain members that are constitutively or inductively regulated with different cellular compartments as a target [32].

HSPs can contribute to tumorigenesis owing to their pleiotropic activities as molecular chaperones. Cancer cells may benefit and alter protein activities involved in the cell cycle, cell growth and apoptosis [32].

Spermatogenesis, during all its developmental stages, is accompanied by the expression of HSPs [33], [34], not an extraordinary fact due to the several epic transformations and cellular differentiation that takes place.

Family	Protein name	Gene symbol	Gene name	Synonyms	Location	Comments
DNAJ	DnaJ homolog subfamily B member 1	*DNAJB1*	DnaJ heat shock protein family (Hsp40) member B1	HSP40	Cytosol	Co-chaperones with HSP70 to enhance ATP activity
Chaperonin	60 kDa heat shock protein	*HSPD1*	Heat shock protein family D (Hsp60) member 1	HSP60; GroEL	Mitochondria	Constitutive/Inducible. Protein import and macromolecular assembly, prevents misfolding and facilitates the correct folding of imported proteins
HSP70	Heat shock 70 kDa protein 1A	*HSPA1A*	Heat shock protein family A (Hsp70) member 1A	HSP70	Cytosol/Nucleus	Stress inducible, involved in folding, transport of new polypeptides and has a role in protein quality control system
	Endoplasmic reticulum chaperone BiP	*HSPA5*	Heat shock protein family A (Hsp70) member 5	GRP78; BiP	ER	Protein folding and quality control in the endoplasmic reticulum lumen
	Heat shock cognate 71 kDa protein	*HSPA8*	Heat shock protein family A (Hsp70) member 8	HSC70	Cytosol/Nucleus	Constitutive. Role in protein quality control system
	Stress-70 protein, mitochondrial	*HSPA9*	Heat shock protein family A (Hsp70) member 9	GRP75; mtHSP70	Mitochondria	Role in mitochondrial iron-sulfur cluster biogenesis; regulates erythropoyesis
HSP90	Heat shock protein HSP 90-alpha (HSP90)	*HSP90AA1*	Heat shock protein 90 family alpha class A member 1	HSP90; HSPC1	Cytosol/Nucleus	Constitutive/Inducible. Role in signal transduction, mantainance and refolding of proteins, regulation of heat shock response, role in cell cycle and proliferation
	Endoplasmin	*HSP90B1*	Heat shock protein 90 beta family member 1	GRP94; GP96	ER	Processing and transport of secreted proteins

Table I-1. Brief summary of the nomenclature, location and function of the principal HSPs

Taking into account the importance of HSPs in somatic cells it would not be surprising they exert a key role in germ cells as well. This thesis is going to focus mainly in three HSPs: HSP60, HSP70 and HSP90.

Cellular stress factors can induce a conserved cell defense mechanism, the heat shock response (HSR), what in the end is a strategy against misfolded proteins induced by harmful factors, such as heat shock (Figure I-1). The HSR mainly involves the expression of HSPs. The activation of HSP is basically achieved to the heat shock transcription factor 1 (HSF1), that functions as the central regulator of the HSR. HSF1 is a stress-inducible DNA-binding transcription factor that plays a central role in the transcriptional activation of the HSR. In basal conditions, HSF1 is found inactive in a monomeric form in the cytoplasm as a part of a multichaperone complex. Upon stimuli, HSF1 undergoes translational modifications, like phosphorylation or acetylation, and trimerization. It translocates to the nucleus and activates HSP gene transcription binding to site-specific heat shock elements (HSEs) present in the promoter regions of HSP genes. Activation is reversible, and during the attenuation and recovery phase period of the HSR, returns to its unactivated form. In this situation HSP are over-expressed, as a result of an absence of stress it and they bind directly to the HSF1 trans-activation domain resulting in its suppression (Reviewed by [30], [35], [36]).

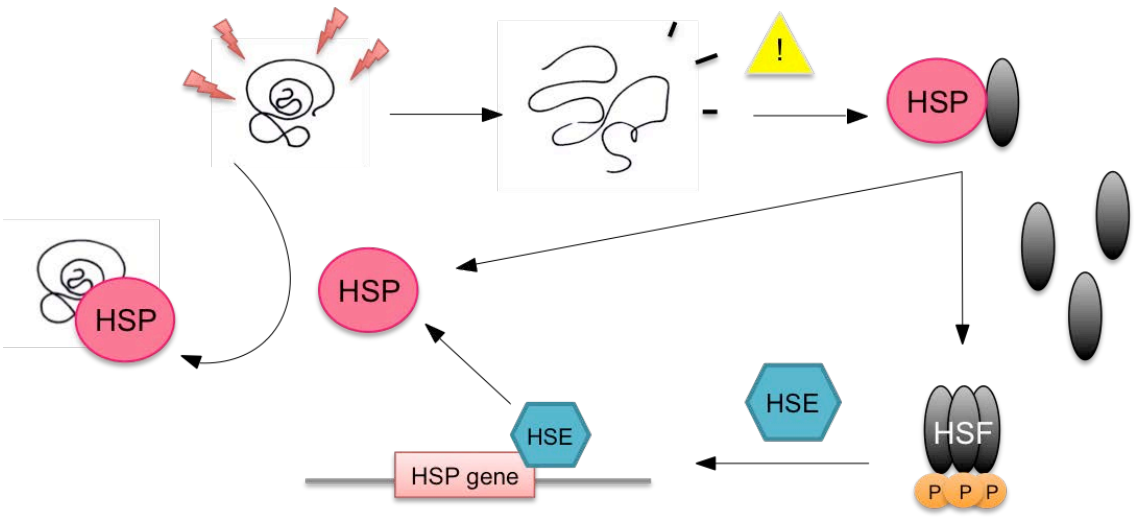


Figure I-1. Basic scheme of HSR activation. Presence of denatured proteins stimulates the dissociation of the HSP-HSF complex. This complex limits the transcription process in the absence of aberrant proteins; while HSP is free to bind to denatured proteins, HSF is being activated through phosphorylation and trimerization in order to produce

more HSPs. Activated HSF can now bind to the heat shock element (HSE). HSE is a part of the promoter region of the HSP gene and more HSPs end up being produced. Newly formed HSPs are able to bind more denatured proteins.

In general, HSPs are expressed intracellularly, however some can be extruded to the cell surface. HSP60, HSP70, HSP90 and endoplasmin have been found in the extracellular medium (reviewed by [31]).

2.1. Heat Shock Protein 60 (HSP60)

Mammalian HSP60 is largely located in the mitochondrial matrix, where it plays a role in macromolecular assembly and might facilitate the refolding of mitochondrial proteins, therefore facilitating the proteolytic degradation of misfolded or denatured proteins in an ATP-dependent manner. The chaperon function of HSP60 during substrate binding and ATPase activity is regulated through its association with HSP10 .

In humans, HSP60 has been detected in seminal fluid and human testis, localized only in spermatogonia, primary spermatocytes, Sertoli and Leydig cells [34], [37]. HSP60 present in the endometrium and oviduct epithelial cells was shown to bind to human spermatozoa, affecting signaling pathways and capacitation [38], and endogenous HSP60 was detected in mature, ejaculated spermatozoa [38]–[40].

Widely studied in rodents, HSP60 is located also in spermatogonia, primary spermatocytes, Sertoli and Leydig cells and mature spermatozoa, which reacquire the protein as they go through the caput epididymis. It is also found on the acrosome of mouse epididymal sperm, in the epididymal epithelium and dense bodies [41]. Moreover, HSP60 and HSP90 are translocated to the sperm surface and phosphorylated on tyrosine residues during capacitation of mouse sperm. All the spermatozoa capable of binding zona pellucida have this pattern of phosphoprotein localization [41], [42]. This differential expression pattern, possibly, it is due to HSP60 carries out distinct functions during the sperm maturation process.

HSP60 has also been shown to be present in mature spermatozoa in bovine, boar, stallion, dog and cat species [43]–[45]. In bovine, has been demonstrated its presence at the surface of the oviduct epithelial cells and its transference to spermatozoa [43].

HSP60 plays an autoantigen role in many autoimmune diseases (adjuvant arthritis in rats, rheumatoid arthritis in humans, insulin-dependent diabetes mellitus in mice, and systemic sclerosis in humans) and pathogenic infections for the high homology between its bacterial and mammalian sequence (reviewed by [46]).

2.2. Heat Shock Protein 70 (HSP70)

Most proteins of the HSP70 family are cytosolic and nuclear, like the heat-inducible form HSP70 or the constitutively expressed HSC70 [47], while mtHSP70 is a mitochondrial protein and GRP78 is localized in the endoplasmic reticulum. The HSP70 isoform is expressed at low levels in normal cells meanwhile its expression is enhanced in stressed cells. The primary function of HSP70 is to transiently bind to newly synthetized polypeptides or denatured peptide sequences, preventing their aggregation and helping them to (re)fold into a functional state, preserving protein conformation. The inherent ability of HSP70 to stabilize partially folded proteins allows it to assist in the transmembrane transport of proteins, and in their assembly into macromolecular complexes [48].

HSP70 has been demonstrated to be up-regulated in response to hyperthermia and to protect cells by refolding denatured proteins, removing the damaged proteins (Welch and Suhan, 1986), and by blocking apoptosis (Mosser *et al.*, 1997; Samali & Orrenius, 1998).

The highly differentiated sperm cells have a residual cytoplasm deprived of translational machinery. Therefore, spermatozoa HSP70 pool can be only synthetized during spermatogenesis. HSP70 has been identified during spermatogenesis in mouse, rat, bull, boar and humans [49]–[54] and in mature sperm in mouse, humans, bull, boar, stallion, dog and cat [39], [45], [50], [53], [55],

[56]. HSP70 protein also persists in ejaculated boar and bovine spermatozoa and is subjected to subcellular translocation during capacitation and acrosome reaction [53], [56]. Moreover, the presence of a specific antibody against HSP70 diminishes the fertilization rate in vitro in bovine [57], which strongly supports its importance in fertilization events.

Testis-specific HSP70 form, HSPA2 (HSP70-2), was originally identified in experiments designed to assess the effects of heat shock on protein synthesis in the germ cells of male mice [49], [50]. It has been found that the disruption of *HSP70-2* gene resulted in failed meiosis, germ cell apoptosis and male infertility [58]. The constitutive form HSC70 accumulates during spermatogenesis in mouse [49], [50]. The expression of GRP78 has been shown during spermatogenesis in mouse [59], [60] and human [60].

2.3. Heat Shock Protein 90 (HSP90)

The HSP90 family is already extremely abundant without a presence of a stressful factor, and makes up 1-2% of cytosolic proteins [61]. The HSP90 family can be divided into five subfamilies: cytosolic HSP90A, endoplasmic reticulum HSP90B, chloroplast HSP90C, mitochondrial TNFR associated protein (TRAP) and bacterial high temperature protein G (HtpG). The cytosolic is the largest and the best-studied group. Mammalian cells contain four HSP90 homologs: two cytosolic (constitutively expressed HSP90AB1 and heat-inducible HSP90AA1), mitochondrial TRAP1 and the endoplasmatic reticulum associated protein GRP94.
HSP90 works as a homodimer and contains three relevant domains:

a. Adenine binding N-terminal domain
b. Charged middle linker region, with high affinity for co-chaperones an client proteins
c. C-terminal dimerization domain, that contains an EEVD motif enabling the binding to tetratricopeptide repeat-binding (TRP) domain of3 co-chaperones.

Levels of HSP90 in cells are dependent on the heat-shock response regulator HSF1 (heat-shock factor 1), which is submitted to a complex of regulatory

processes. Additionally, under physiological conditions, HSP90 functions as a part of a complex of co-chaperons and client proteins like calmodulin, transcription factors, actin, tubulin, several kinases and some receptor proteins. Complexes aimed at protecting microtubules, maturation of key signaling proteins including regulatory kinases, steroid hormone receptors and transcription factors, and are also implicated in protein folding and degradation. Under heat shock stress, HSP90 can be associated to protein kinases and steroid receptors, which may mediate nuclear transport [62], [63].

Hsp90 has been studied in the sperm of diverse mammals including rat [64], mouse [42], [65], [66], boar [44], stallion, dog, cat [45], rabbit [67], and humans [68], [69]. HSP90 has been suggested to play an important role in sperm motility [70] and to enhance fertilization capacity [64].

The fact that a protein like HSP90 is so abundant suggests that the lack of it can lead to major errors or failures, such as meiotic arrest resulting in germ cell loss and reduction in testicular size resulting in sterile mice [71].

Exposing cells to stress may lead to aberrant protein conformation. The accumulation of these misfolded proteins can be a threat for our cryopreserved germ cells. Promoting this efficient quality control system to refold or destroy misfolded proteins may be a strategy to protect cells from the harsh process of cryopreservation.

2.4. Studying HSPs in oocytes and embryos

In 1983, Bensaude *et al*. [72] first characterized constitutive and inducible forms of HSP70 in 2-cell mouse embryos, and it was not until 1996 those same proteins were described in bovine [73].

The study of HSPs and heat stress (HS) on bovine oocytes and embryos has been approached from three different points:

a. *Heat stress as an economical factor:* The impact of heat stress during hot seasons has been broadly studied for its influence on animals fertility, thus ultimately for its economic repercussion on stockbreeding. In the actual context of global warming and climate change HS is a topic that continues to arouse interest. In the case of summer HS, hyperthermia affects approximately 60% of world cattle population [73]–[78].

b. *Thermotolerance:* To induce a cellular resistance to stress through a controlled heat shock treatment, commonly called a sublethal stress. The data around this aspect is quite divergent, as the possibilities in temperatures and time exposure are enormous. When HS was applied during maturation, oocytes surpassed the first cellular division with a similar success to controls [73], [79]–[85]. In terms of blastocyst outcome, HS could produce no effect [80], [86] or be detrimental for embryo development [79], [83], [86]–[90]. Interestingly, in some cases they got the right stimuli and HS did increase developmental rates in bovine [85] and pig [91]. In these cases, authors hypothesized HSPs generated a protective role on oocyte and embryo development.

c. *HSPs induction:* the study of the induction of HSPs can be divided in two groups, one, the works that assess the use of a controlled heat stress to study the modulation of HSPs [73], [79], and in the other hand, investigations that measure the levels of HSPs as a biomarker or indicator of cellular stress under a certain treatment [92]. In both cases, the HSP studied the most is HSP70, measuring either protein levels or its transcript (*HSPA1A).* The spontaneous expression of *HSPA1A* family during early development [93], [94] offers the opportunity to identify cellular events taking place in early embryos.

These three ways to approach HSPs research can converge easily. A study made by Souza-Cácares *et al*. (2019) [95] showed how environmental conditions like heat and humidity can modulate the presence of HSP70 on different cattle breeds. The presence of HSP70 and HSP90 was assessed in two different breeds,

one of them, native and thought to be naturally adapted to these particular harsh conditions in Brazil. Independently from variations on climate conditions, native cows presented lower levels of HSP90 and higher of HSP70, even doubling that compared to the other breed. When the heat and humidity increased, oocyte quality was clearly affected in the unadapted breed, accompanied with an increase of HSP70. Surprisingly, under this conditions oocyte quality improved in native cattle, what was followed with a reduction of HSP70. It seems clear then, that an adaptation to changes in temperature conditions is possible through the action of HSPs. Another work showed how many HSP genes varied during different seasons in cattle and buffalo. The expression was higher in summer and winter when compared to the thermoneutral season. *HSPA1A* and *HSPA1B* presented maximal levels in summer and winter, while *HSPA8* (constitutive form of HSP70) levels did not change. *HSP60* and *HSP10* were the second most abundant HSPs. Buffalo presented higher levels of HSPs when compared to cattle breeds, what, together with the elevated levels of *HSF1,* was indicative of a grater capacity for adaption or heat tolerance [96].

Other members of the HSP70 family can be also modulated by heat, like HSP70-4, a protein associated with ribosomes and involved in protein folding and ATPase activity [97]. Summer-collected oocytes, with worse maturation rates, presented a down-regulation of *HSPA14* (HSP70-4), compared to those from winter.

In vitro procedures still need to be improved as they differ from the *in vivo* ones[98], [99]. The microenvironment created during those procedures will affect the oocyte and embryo gene expression [100], [101]. Evaluating transcript levels for *HSPA1A* has been broadly used as an indicator of cellular stress [12], [102], [103], as it can be can be modulated through treatments during culture. Changes in that microenvironment can be reflected later on in the embryo gene expression. Analyzing levels of *HSPA1A*, is a way to test supplements for culture. Addition of melatonin resulted in better blastocyst rate and in increased expression of *HSPA1A*, accompanied with an increase of *BCL2L1*, an anti-apoptotic gene, and a reduced expression of *CASP3* (caspase 3), an activator of apoptosis [104] .

3. IL-6 FAMILY OF CYTOKINES

Interleukin (IL) -6 familiy of cytokines play integral roles in numerous physiologic and pathophysiologic processes. Their pleiotropic members are engaged in homeostasis, immunity, infection and inflammation. Their functions are partially overlapping and redundant, but they can also be involved in well-defined and singular actions. The classic IL-6 family of cytokines consisted of eight members: IL-6, IL-11, leukemia inhibitory factor (LIF), IL-27, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), and cardiotrophin-like cytokine factor 1 (CLCF1). Later, two more cytokines, IL-35 and IL-39, were added to the family (Collison *et al*., 2012; Wang *et al*., 2016). What agglutinates all members is a common core structure, a signal transducer in their receptor complexes, the glycoprotein 130 (GP130) (Figure I-2). Formation of cytokine signaling complexes on the cellular membrane triggers activation of the intracellular Janus kinase, thereby activating JAK/STAT, MAP-kinase and PI(3)-kinase pathways (reviewed by [105]–[108]). The activation of specific members of each protein family (signal transducers) along the signaling pathway will be determined by each cytokine. This is, LIF will activate mainly STAT3 and STAT1, which in turn will activate SOCS3. This is the "fine-tuning" of the signal transduction.

Cytokines are known to play crucial roles in inflammation. It has been suggested that follicular development and ovulation are not only similar to an inflammatory reaction but are associated with the expression of specific cytokines. LIF, IL-6 and IL-11 have been described to be important for the reproductive function.

IL-6 was discovered as a B-cell differentiation factor [109], [110]. It is produced by a number of cell types that play important roles in inflammation [111], immunity [112] and hematopoiesis [113]. IL-6 has been detected in human follicular fluid [114]–[116] and granulosa cells [115] and its transcripts are present from zygote up to blastocyst stage [117]. It seems to be involved in cumulus expansion [118]–[120] and implantation, given that an alteration of its

expression, elevated or insufficient, leads to miscarriage (reviewed by [121]). IL-6 produced during follicular growth and maturation plays an important role in angiogenesis and in the corpus luteum function [122]. This and other cytokines are found increased in serum and follicular fluid of women with endometriosis [123]–[125]. A study performed by Banerjee *et al.* (2012)[126], showed how mouse oocytes treated with elevated levels of IL-6, those normally associated with endometriosis and pelvic inflammation, caused dose-dependent deterioration of microtubules and chromosomes, what may result in a reduced fertility. In contrast, IL-6 appears to be involved in the proper embryo development [127]. Parthenotes cultured with IL-6 increased in blastocyst rate and reduced in apoptosis [128] Also, embryo supplementation leads to an increase of ICM and total cell number[117], [128] .

IL-11 is an anti-inflammatory cytokine that was first identified and characterized in human fibroblasts [129] . In mammalian species is a non-glycosylated 19 kDa protein made up of 178 amino acids. It was initially described as a growth factor involved in hematopoiesis, causing megakaryocyte differentiation, synergizing with other factors [130] . Within the reproductive function, IL-11 is mainly implicated in implantation, hatching and decidualizatio[131] . Low doses of IL-11 improve hatching and attachment of blastocysts, but both high and low doses impair decidualization [132] . Female mice lacking the IL-11R are infertile due to a defect in decidualization in response to the implanting blastocyst [133], [134] . It is the IL-11 classic rather than IL-11 trans-signaling (soluble receptor form) the one that contributes to female fertility in mice [135]

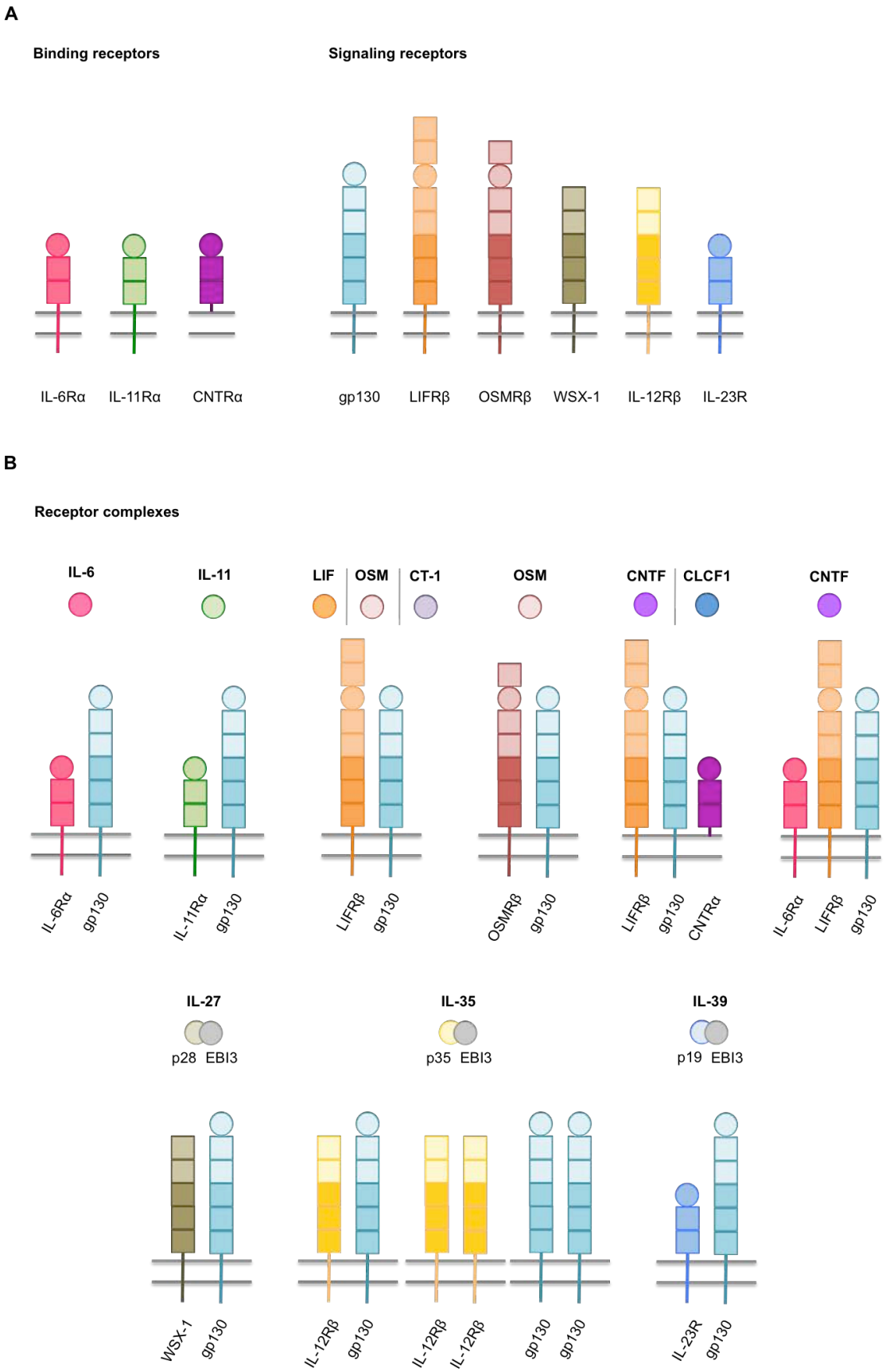


Figure I-2. Receptor complexes of IL-6 family of cytokines. A) The IL-6 family cytokines act via four different ligand-binding receptors and six different signaling

receptors. B) Molecular set-up of the receptor complexes. The signal transducer GP130 is shared for all ten members. Some ligands can bind to different complexes, like OSM, CNTF and IL-35. IL-6: Interleukin-6; IL-11: Interleukin-11; LIF: leukemia inhibitory factor; OSM: oncostatin M; CT-1: cardiotrophin-1; CNTF: ciliary neurotrophic factor; CLCF1: cardiotrophin-like cytokine factor 1; IL-27: Interleukin-27; IL-35: Interleukin-35; IL-39: Interleukin-39.

3.1. Leukemia Inhibitory factor

Cytokines and growth factors are naturally found in follicular fluid, promoting oocyte growth and survival, thus affecting the possible fertilization and embryo development. One of these cytokines is leukemia inhibitory factor (LIF), a 20kDa glycoprotein. LIF is synthesized as a 202 amino acid precursor that is post-transcriptionally processed into a 20 kDa form by removing 22 amino acids from its N-terminus. Similarly to other IL-6 family cytokines, LIF exists as a compact four-helix bundle in an up-up-down-down configuration (reviewed by Nicola & Babon, 2015).

Cytokines and growth factors are naturally found in follicular fluid, promoting oocyte growth and survival, thus affecting the possible fertilization and embryo development. One of these cytokines is LIF. LIF was first described to induce differentiation in M1 murine myeloid leukemic cells (Gearing *et al.*, 1987) and to maintain totipotency in embryonic stem cell lines (Williams *et al.*, 1988). LIF has been detected in human follicular fluid [136] , granulosa cells[137] and transcripts for LIF were found from matured oocytes up to 16-cell stage in bovine [138] . It plays a crucial role in follicular development and ovulation, since it has been shown to promote primordial to primary transition in rats [137] , to coordinate follicular growth and ovulation in mice [139] and to preserve cell function and increase follicular development in both vitrified and non-vitrified ovaries [140] .

Several studies have been carried out on the use of LIF as a supplement to the maturation media in mouse and livestock species. It has been reported to promote oocyte maturation in mice [141] , bovine [142] , pig [143] and slightly in goat [144] , and its effects persisted until early developmental stages, even improving the blastocyst rate [141], [142] . When supplemented in the culture

media it improved cleavage rate in sheep [145], [146] , early developmental stages [147], [148] and developmental competence in mice [149] . In terms of blastocyst quality, LIF treated embryos had better hatchability [133], [148], [150], and embryos were described to increase in total cell number in mouse [149] , bovine [142], [151] , buffalo [150] and sheep, also presenting a grater proportion of inner cell mass number [146] . LIF has also been described as an important factor for blastocyst implantation [152], [153], trophoectoderm proliferation [154] and invasion [155] .

LIF receptor consists of two signaling chains: LIFRβ and GP130, any of which have intrinsic kinase activity within its intracellular domains, reason why they associate with JAK family of tyrosine kinases [156] .

After LIF or any other cytokine bind to the receptor complex, GP130 (and LIFRβ) activates Janus kinase (JAK) family of tyrosine kinases by transphosphorilation (Figure 3). Once JAK are catalytically competent, a cascade is initiated stimulating three different signaling pathways: JAK/STAT, MAP-kinase and PI(3)-kinase, activating target genes involved in differentiation, survival, apoptosis and proliferation.

a. **JAK/STAT3:** Once JAK are trans-activated, they in turn phosphorylate the cytoplasmic tails of the receptors to create docking sites for STAT3 and STAT1. The STAT family (signal transducers and activators of the transcription) is a group of proteins that work as transcription factors and are usually found inactive in the cytosol. Upon activation, they dimerize and translocate into nucleus where they will modulate gene transcription for a specific cytokine (reviewed by [157], [158]). One of the target genes is *SOCS3* (suppressor of cytokine signaling 3), which completes a negative feedback loop by inhibiting STAT proteins. This inhibition is achieved by binding to specific sites on JAK and GP130 to induce ubiquitination. Also, when binding to JAK its catallitic activity is inhibited. The binding site for SOCS3 on GP130 and LIFRβ is a phosphotyrosine motif that usually recruits SHP2 to activate MAPK-pathway. Therefore, a part from inhibiting STAT3

signaling, SOCS3 shuts down MAPK-pathway by competing with SHP2 for its binding site.

b. **PI(3)-KINASE:** PI(3)-kinase is an enzyme that catalyzes the formation phosphatidylinositol (3,4,5)-triphosphate from phosphatidylinositol (4,5)-diphosphate which in turn activates a number of downstream effectors. An association between p85 and JAK is necessary for signaling activation. Then, p85 activates serine/threonine AKT leading to a signaling cascade.

c. **MAPK:** as described above, following the receptor complex activation, phosphorylated receptor chains recruit the phosphatase SHP2. Once SHP2 is activated, Ras/Raf signaling is induced leading to the activation of MAPK. Transcription factors like Elk, c-Fos or c-Jun will be activated (reviewed by [159]).

Tscherner *et al.* (2018) revealed STAT3 as a transcriptional activator of miR-21 in the bovine cumulus-oocyte complex (COCs) after LIF exposure. miR-21 seems to play an important role in the ovary, its expression increases during the estrus, is the most abundant miRNA in bovine cumulus cells and it has been described as a key factor in ovine folliculogenesis (reviewed by [160]).

Chapter II

Effect of heat stress during *in vitro* maturation on developmental competence of vitrified bovine oocytes

1. ABSTRACT

HSP70 plays a crucial role as intracellular cytoprotectant and molecular chaperone. A phenomenon of heat stress (HS) leads to production of these proteins that could be beneficial to cells during cryopreservation, which is also a stressful process for the cell. This study aimed to evaluate the protective effect of exposure of bovine oocytes to moderate HS during in vitro maturation prior vitrification. First, oocytes were subjected to HS (41.5° C for 1 h) at 0, 4, 8, 12, and 16 of IVM. Oocytes cultured for 20 h served as control group. Presence of HSP70 was detected at 20h by immunofluorescence. HSP70 expression was significantly higher when oocytes were subjected to HS at 8 h of IVM. Next, oocytes were distributed in 4 groups: Control: IVM oocytes; VIT: oocytes vitrified/warmed at 20 h of IVM; HS: oocytes subjected to HS at 8 h of IVM; HS-VIT: oocytes subjected to HS at 8 h of IVM and vitrified/warmed at 20 h of IVM. Oocytes were fertilized at 24 h of IVM and cleavage and blastocyst yield were assessed. No significant differences were observed among treatments when cleavage rate was evaluated. However, fresh control and HS oocytes resulted in a significantly higher blastocyst rate when compared to VIT and HS-VIT groups although no significant differences within fresh or vitrified groups were observed. In conclusion, HS did not have a negative impact on the oocyte competence but HS applied before vitrification, offered no benefits for embryo development.

2. INTRODUCTION

Vitrification is a cryopreservation technique especially useful when dealing with in vitro produced or micromanipulated oocytes. The oocytes of many mammalian species have been successfully vitrified. However, in bovine species, the low fertilization rates and developmental competence of cryopreserved oocytes still need to be improved. Although results reporting blastocyst development exceeding 10% are common, variability remains high, and a standard method for bovine oocytes remains to be established [161] .

Recent studies have demonstrated that well-defined and properly applied stress, such as high hydrostatic pressure or osmotic, heat or oxidative stress, could increase tolerance of gametes and embryos to several in vitro and in vivo procedures [162] . When cells are subjected to various stress factors, they increase the production of a group of proteins called heat shock proteins (HSP). HSP are highly conserved chaperones expressed as a response to a raise in temperature and to other environmental factors to protect cells and they seem to be beneficial to the cells during cryopreservation, which is also a stress-inducing procedure [162] .

Oocyte synthesizes HSP70 in response to a moderate HS applied at different moments during in vitro maturation. Next, we studied whether this presumptive accumulation of HSP70 prior to vitrification/warming improved their cryotolerance and developmental competence following fertilization.

3. MATERIALS AND METHODS

3.1. *In vitro* maturation, fertilization and embryo culture

In vitro maturation, fertilization and embryo culture procedures have been described elsewhere [163] .

2.1. Oocyte vitrification and warming

At 20 h of IVM, partially denuded oocytes were vitrified/warmed as described by [164]. After warming, oocytes were transferred back into the maturation dishes to allow them to mature up to 24 h.

2.2. Immunofluorescence for HSP70 detection

Oocytes were denuded of cumulus cells by gentle pipetting and fixed in 4% paraformaldehyde (PFA, 1 h, 38.5ºC), permeated (2.5% Triton-X100, 20 min, 38.5ºC) and blocked (3% BSA, 30 min, 38.5ºC). Oocytes were incubated with mouse Anti-Hsp70 antibody [5A5] (Abcam, Cambridge, UK) overnight at 4ºC, incubated with goat anti-Ms AlexaFluor® 568 (1:300) for 1 h at RT. Oocytes were

transferred to droplets of mounting medium containing DAPI (Vectorlabs, Burlingame, CA, USA), coverslipped and fluorescence intensity of HSP70 was assed under a confocal laser scanning microscope. ImageJ was used to quantify the integrated density (fluorescence intensity).

2.3. Experimental design

First, COCs were randomly allocated in six groups. COCs were heat stressed (1 h at 41.5ºC) at 0, 4, 8, 12 and 16 h of IVM. Oocytes cultured for 20 h without HS served as control group. After 20 h of IVM, oocytes from all groups were fixed in PFA to evaluate HSP70.

Second and based on the results obtained from the previous experiment, COCs were distributed in 4 groups: Control: IVM oocytes; VIT: oocytes vitrified/warmed at 20 h of IVM; HS: oocytes subjected to a HS at 8 h of IVM; HS-VIT: oocytes subjected to a HS at 8 h of IVM and vitrified/warmed at 20 h of IVM. Oocytes were then fertilized at 24 h of IVM. Cleavage rates were recorded at 48 h post-insemination and blastocyst yield determined on days 7 (D7) and 8 (D8) post-insemination.

2.4. Statistical analysis

All of the experiments were performed six times. Statistical significance of the fluorescence intensity was inferred from repeated measures ANOVA. Oocyte survival, embryo cleavage rates and blastocyst yield were analyzed by a two-way ANOVA. The level of statistical significance was set at $P< 0.05$.

3. RESULTS

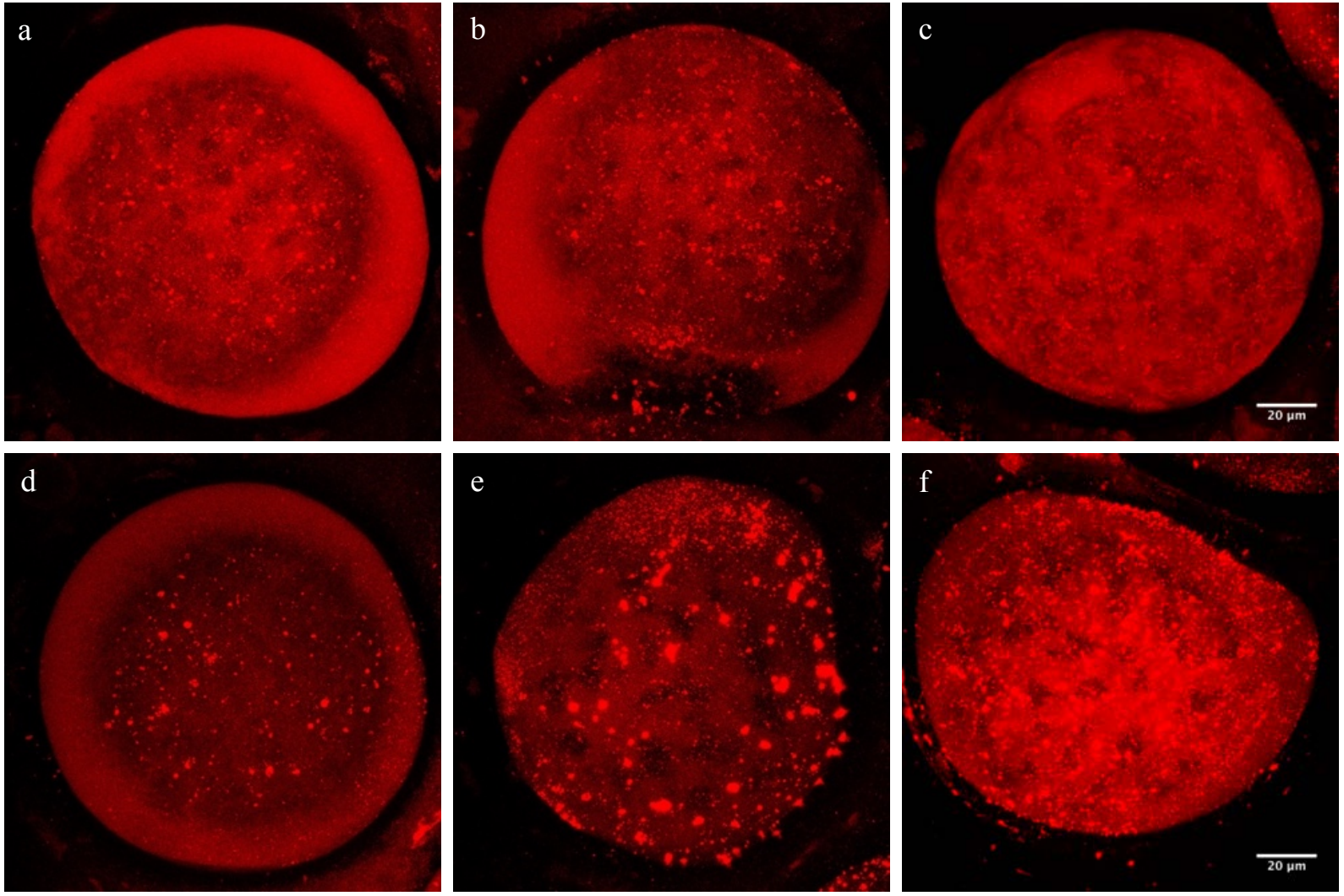
Significantly, higher levels of fluorescence were observed when HS was applied at 8 h of IVM when compared to the control group (20h) while no significant differences in fluorescence intensity were observed among the other groups (Table 1 and Figure 1).

Treatment	n	Fluorescence intensity
0 h	22	1,44E+06 ± 1,12E+05[a,b]
4 h	25	1,30E+06 ± 1,13E+05[a,b]
8 h	30	1,52E+06 ± 7,70E+04[a]
12 h	30	1,45E+06 ± 6,22E+04[a,b]
16 h	24	1,30E+06 ± 8,61E+04[a,b]
20 h (control)	33	1,14E+06 ± 9,19E+04[b]

Table II-1. HSP70 fluorescence intensity. Comparison of HSP70 relative fluorescence intensity of bovine oocytes subjected to HS treatment at different moments of IVM.Data show the mean ± SEM of relative unities. Values without a common superscript differ ($p < 0.05$). .

No significant differences were observed among treatments when cleavage rate was evaluated. However, fresh control and HS oocytes resulted in a significantly higher blastocyst rate when compared to VIT and HS-VIT groups. However, no significant differences within fresh or vitrified groups were observed, regardless of the HS treatment Table 2).

Figure II-1. Immunofluorescence detection of HSP70 in bovine oocytes. a-e: oocytes exposed to HS at 0, 4, 8, 12, 16 and of IVM, respectively; f: control: 20 hours of IVM. Red color: HSP70 (AlexaFluor® 568). *[Next page]*


a
b
c
20 µm
d
e
f
20 µm

				Blastocyst (%)	
Treatment	**n**	**Survival**	**Cleavage (%)**	**D7**	**D8**
Fresh control	268	181	67.0 ± 5.8	19.4 ± 3.2[a]	21.23 ± 2.3[a]
HS	195	136	82.4 ± 3.0	13.8 ± 2.7[a]	15.41 ± 3.6[a]
Vitri	132	95	64.2 ± 4.1	4.3 ± 0.3[b]	5.28 ± 1.0[b]
Vitri-HS	160	73	65.0 ± 2.3	1.3 ± 0.8[b]	2.61 ± 1.1[b]

Table II-2. Embryo development. Comparison of development rates of bovine oocytes subjected to HS treatment before vitrification/warming.Data show the mean ± SEM. Within a column, values without a common superscript differ ($p < 0.05$).

4. DISCUSSION

In the present study, a significant increase in HSP70 contents was successfully triggered in bovine oocytes by a moderate treatment of HS when applied at 8h of IVM. Higher relative fluorescence intensity for HSP70 was detected at 8h of IVM when compared to the control group, whereas the application of the procedure at 0h, 4h, 12h and 16h of IVM did not allow a significant increase. Previous studies already observed a substantial increase of protein synthesis (approximately three-fold) in the time interval from 6 to 10 h of IVM [165] , which occurs in parallel with the onset of germinal vesicle.

From the results of this study at Experiment 2, it is shown that a moderate HS of 41.5ºC for 1 hour applied at 8h of IVM is not detrimental for oocyte competence in terms of blastocyst yield. This is in concordance with the study from [80] who after applying a similar HS procedure at 20-22h of IVM did not find deleterious effects on development to the blastocyst stage.

Therefore, the procedure of HS described in this study is not over the limit of tolerance of bovine oocytes and we suggest that it is able to stimulate HSP70 oocyte contents as shown in Experiment 1. However, these circumstances did not

allow coping the exigent demands of vitrification and oocytes exposed to HS did not show better blastocyst yield than control vitrified/warmed oocytes. It is well known that stress-induced proteins may either inhibit or even promote cell death pathways [166] . Although HSP70 is considered to be an antiapoptotic protein, a pro-death role has also been ascribed to Hsp70, reducing or facilitating the activation of the apoptotic cascade in a delicate equilibrium [166] . Further experiments are needed to assess the influence of increasing or reducing HSP70 cellular contents over resistance to vitrification.

Chapter III

Effects of IL-6 family members on specific miRNA expression during *in vitro* oocyte maturation

1. ABSTRACT

Cytokines play crucial roles in inflammation. Follicular development and ovulation have many similarities with inflammatory processes and the expression of specific cytokines is actually required for ovulation. Members of the interleukin (IL-6) family of cytokines including leukemia inhibitory factor (LIF), IL-6 and interleukin-11 (IL-11) are important for reproductive function. LIF participates in follicular growth, oocyte maturation and implantation, IL-6 plays key roles in the corpus luteum and cumulus expansion and IL-11 facilitates implantation. IL-6 cytokines stimulate the intracellular Janus kinase/signal transducer, thereby activating JAK/STAT, MAP-kinase and PI(3)-kinase pathways. This alters downstream expression of genes and microRNAs (miRNAs) in oocytes and follicular cells, creating a microenvironment that improves oocyte quality and competency. Understanding cytokine-mediated changes in gene and miRNA expression may inform strategies to improve oocyte cryotolerance during vitrification. We therefore characterized the effects of LIF, IL-6 and IL-11 on miRNA expression in bovine cumulus-oocyte complexes matured *in vitro*. We assessed the expression of *miR-21*, *miR-155*, *miR-34c* and *miR-146a*, miRNAs previously implicated in oocyte maturation and cumulus expansion. Oocytes were distributed in five groups: GV (germinal vesicle), Control (matured in TCM199 + 20% FBS + FSH + LH + E2), LIF (matured in TCM199 + 25 ng/mL LIF), IL-6 (matured in TCM199 + 10 ng/mL IL-6), IL-11 (matured in TCM199 + 5 ng/mL IL-11) and non-supplemented (oocytes matured in TCM199). After 24h of IVM, cumulus cells were stripped from oocytes and both cumulus cells and oocytes were collected for miRNA extraction and qPCR analysis. The effects of treatment were analyzed by one-way ANOVA followed by a Sidak test ($p<0.05$). MicroRNA-21 expression was significantly higher in cumulus cells from the control (FBS) and LIF groups and was higher in LIF-treated oocytes compared to TCM199 alone. IL-11 treatment increased miR-146a expression in oocytes while no significant differences were observed in the levels of miR-146a in cumulus cells. In cumulus cells, miR-155 was significantly higher in controls, compared to oocytes, where no differences were observed between groups. The presence of cytokines during maturation had no effect on miR-34c expression in cumulus cells or oocytes in any group. In conclusion, the addition of LIF to the

maturation media may improve oocyte quality through increased expression of miR-21, which has been shown to have anti-apoptotic effects. Further studies to evaluate the potential cryoprotective effects and mechanisms of action of LIF on bovine oocytes are warranted.

2. INTRODUCTION

Cytokines were originally characterized as key participants in inflammation but are now recognized to play other roles many in processes such as follicular development and ovulation [116], [119] . Three members of the interleukin-6 (IL-6) family of cytokines, leukemia inhibitory factor (LIF), IL-6) and IL-11, are particularly important in reproductive contexts. The IL-6 family of cytokines share a common signal transduction receptor, the glycoprotein 130 receptor (GP130) (reviewed by [105]). Formation of cytokine signaling complexes at the cellular membrane triggers activation of the intracellular Janus kinase/signal transducer, thereby activating JAK/STAT, MAP-kinase and PI(3)-kinase pathways [105], [108], [159].

LIF was first described as a protein factor that induces differentiation in M1 murine myeloid leukemic cells [167] and maintains totipotency in embryonic stem cell lines [168] . LIF has been detected in human follicular fluid [136] and granulosa cells [137] , and LIF-transcripts have also been found in matured bovine oocytes and early embryos (up to 16-cell stage) [138]. This factor appears to play a crucial role during follicular development and ovulation, since it has been shown to promote primordial to primary transition in rats [137] , to coordinate follicular growth and ovulation in mice [139] , and to preserve cell function and increase follicular development in both vitrified and non-vitrified ovaries [140] . Several studies have investigated the use of LIF as a supplement to the maturation media in mouse and livestock species. In these studies, LIF was found to promote oocyte maturation in mice [141] , bovine [142] , pig [143] and goat [144] , and its effects persisted until early developmental stages, even improving the blastocyst rate [141], [142] . When added to the culture medium, LIF improves cleavage rates in

sheep [145], [169] , [146], and early developmental stages [147], [148] and enhances developmental competence in mice [149] . In terms of blastocyst quality, LIF treated embryos have higher hatching rates [148], [150] , a greater total cell number, in mouse [149] , bovine [142], [151] , buffalo [150] and sheep, where a greater proportion of inner mass cells is noted [146] . LIF has also been described as an important factor for blastocyst implantation [152], [153] , trophoectoderm proliferation [154] and invasion [154], [155].

IL-6 was originally identified as a B-cell differentiation factor [109], [110], [170] that is produced by a number of cell types and which plays important roles in inflammation [111] , immunity [112] and hematopoiesis [113] . IL-6 has been detected in human follicular fluid [114]–[116] and granulosa cells [115] , and IL-6 mRNA transcripts are present throughout development from zygote to the blastocyst stage [117] . It appears to be involved in cumulus expansion and implantation [118]–[120] , as alterations in expression levels, either elevated or suppressed, lead to miscarriage (reviewed by [121]). IL-6 produced during follicular growth and maturation plays important roles in angiogenesis and in corpus luteum function [122], [171] . This and other cytokines are found increased in serum and follicular fluid of women with endometriosis [123]–[125]. Interestingly, Banerjee et al. (2012) [126] showed that exposing mouse oocytes to the same high levels of IL-6 found in patients suffering from endometriosis and pelvic inflammation caused dose-dependent deterioration of microtubules and chromosomes, which could result in reduced fertility. In contrast, IL-6 appears to be involved in proper embryo development [127] , as embryo culture with IL-6 increases blastocyst rates and reduces apoptosis in parthenotes [128] , and also augments ICM and total cell number of IVF-embryos [117], [128]. Importantly, the study by [126] contrasts with the other cited work in that IL-6 supplementation is associated with a detrimental effect on overall development.

IL-11 is an anti-inflammatory cytokine that was initially described as a growth factor involved in hematopoiesis, causing megakaryocyte differentiation and synergizing with other factors (reviewed by [130]). With regard to reproductive roles, IL-11 is primarily implicated in implantation, hatching and decidualization

(reviewed by [131] . Low doses of IL-11 improve hatching and attachment of blastocysts, but both high and low doses impair decidualization [132] . Female mice lacking the IL-11R are infertile due to a defect in decidualization in response to the implanting blastocyst [133], [134]. The effect of IL-11 on fertility has been shown to be dependent on the canonical signaling pathway [135] .

Mammalian oocytes normally acquire the capacity to become fertilized and developmental competence during their final maturation within the ovary [172] . Nuclear and cytoplasmic maturation rely on molecules present within the follicular fluid and on interactions with somatic cells surrounding the oocyte, the cumulus oophorus [172] . Tightly regulated communication between the oocyte and cumulus cells is important at various stages, promoting oocyte competency. The exact mechanisms by which cumulus cells influence or contribute to oocyte maturation and fertilization remain unknown, but it is widely recognized that molecules from this microenvironment, including non-coding RNA molecules, move through *zonula adherens*-like junctions [173], [174] .

MicroRNAs are highly conserved, small non-coding RNA molecules 19-24 nucleotides in length that regulate gene expression by targeting mRNA for degradation or by specifically inhibiting translation. The most common pathway of miRNA biogenesis is through processing from a larger transcript, known as a primary-miRNA (pri-miRNA). Pri-miRNAs are normally generated by RNA polymerase II dependent transcription and cleaved by the RNAse III enzyme Drosha, which is the active component of a multiprotein complex known as the microprocessor into ~70 nt precursor-miRNAs (pre-miRNAs). Pre-miRNAs, usually containing a hairpin loop , are transported into the cytoplasm through Exportin-5 dependent pathways where they are further processed into mature miRNAs by a second RNAse III enzyme known as Dicer (reviewed by [175]–[177] . MicroRNAs have been shown to be important regulators of gene expression during oocyte maturation and embryo development [160], [178], [179] . The roles of miRNAs in the growth and maturation of oocytes and their surrounding follicular cells have not been widely characterized. However, through in silico and in vitro examination of specific target genes, it appears likely that these molecules

participate widely in the regulation of apoptosis, proliferation and signaling pathways that are important for follicular growth and oocyte maturation [180] . In particular, the miRNA miR-21 has been observed to change profoundly during oocyte maturation and early embryogenesis [179], [181], [182] . A limited number of studies have suggested potential roles for LIF in the specific regulation of miR-21 expression [160] , but a more comprehensive examination of the roles of the IL-6 family and the involvement in the JAK/STAT signal transduction pathway in the control of miRNA expression in oocytes and cumulus cells has not been undertaken. In order to identify potential roles that miRNAs may play in follicular biology in response to cytokine signaling, the present study aimed to determine whether the IL-6 family members LIF, IL-6 or IL-11 induced changes in the expression of miR-21 and other key miRNAs in bovine cumulus cells and oocyte cultured in vitro as cumulus oocyte complexes.

3. MATERIAL AND METHODS

3.1. Chemicals and suppliers

Unless stated otherwise, all chemicals and reagents were purchased from Sigma Chemical.

3.2. Cumulus-oocyte complexes collection and *in vitro* maturation

For this study, serum-free media for cumulus-oocyte complexes (COCs) collection and maturation was used. The control group was supplemented as described previously [160] . Bovine ovaries were obtained from a local slaughterhouse and transported at 35 °C. Within 2 hours of ovary collection, COCs were aspirated from 2–10 mm follicles using vacuum aspiration. Complexes were aspirated and collected in serum-free, 1 M HEPES-buffered TCM-199 (H-TCM199) collection medium supplemented with 2 IU/ml Hepalene (LEO Pharma, ON, Canada), 1 μg/mL gentamicin and 0.1% polyvinyl alcohol (PVA). COCs were washed twice in H-TCM199 maturation medium supplemented with 22 μg/mL sodium pyruvate, 10

μg/mL gentamicin and 0.1% PVA and randomly assigned to 5 treatment groups: **GV**: immature oocytes at germinal vesicle stage; **Control**: COCs matured in H-TCM199 supplemented with 25 μg/mL FSH, 1 μg/mL LH, 1 μg/mL estradiol (National Institutes of Health (NIH), USA) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA; USA); **LIF**: COCs matured in H-TCM199 supplemented with 25 ng/mL recombinant human LIF (R&D Systems, Minneapolis, MN; USA); **IL-6**: COCs matured in H-TCM199 supplemented with 10 ng/mL recombinant human IL-6 (Gibco, Frederick, MD, USA); **IL-11:** COCs matured in H-TCM199 supplemented with 5 ng/mL recombinant human IL-11 (Abcam, USA); Non-supplemented; **NS**: COCs matured in H-TCM199 supplemented with 10% FBS. Groups of up to 20 COCs were placed in 100 μL drops of maturation medium covered in mineral oil, and cultured in a humidified atmosphere at 38.5 °C and 5% CO_2. After 24 hours, cumulus cells were removed from the oocytes of each treatment mechanically in PBS with 0.1% PVA and pelleted by centrifugation at 600×g and room temperature for 6 minutes. Cumulus free oocytes were treated with 2 mg/mL hyaluronidase to remove any remaining cumulus cells, and washed in PBS-PVA. Cumulus free oocytes and the corresponding cumulus cells of each treatment were immediately flash frozen in liquid nitrogen and stored at −80 °C.

3.3. RNA isolation and quantitative PCR

Total RNA, including small RNA, was isolated using the miRNeasy Micro kit (Qiagen; Mississauga, ON, USA) according to the manufacturer's protocol and as described previously (63). RNA was isolated from pools of 30 COCs as cumulus or oocyte fractions for use with miRNeasy kit. miRNA was extended by polyadenylation then reverse transcribed with qScript microRNA cDNA Synthesis Kit (Quantabio). qRT-PCR was performed with a CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Inc., Hercules, CA) using PerfeCTa SYBR Green SuperMix (Quantabio). cDNAs encoding miRNAs were amplified with a gene-specific forward primer (Table 1) and PerfeCTa Universal PCR Primer (Quantabio). Two μL of cDNA template were used for each reaction. Efficiencies were calculated by standard curve for all primers designed in this study and gene expression was calculated by the efficiency-corrected ΔΔCt method [183] . miRNA were normalized to snRNA U6, which has been previously shown to be suitable for

oocytes [184] and is stably expressed in cumulus cells. In each run, there were three technical replicates from each of the three biological replicates per individual gene. Further, negative controls for the template and for the reverse transcription were also included and amplified by PCR to ensure no cross-contamination. Expression of each miRNA is shown relative to the abundance of that miRNA in the immature oocyte group.

3.4. Statistical analysis

Statistical tests were performed using the statistical package IBM SPSS Version 25.0 for Windows (IBM Corp.; Armonk, NY, USA). Gene expression data collected from qRT-PCR were log2 transformed in order to conduct the appropriate statistical analysis. Data were first checked for normality using the Shapiro-Wilk's test and for homogeneity of variances using the Levene test. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by the post-hoc Sidak's test. The level of significance was set at $P \leq 0.05$.

Primer name	Primer sequence (5'-3')	Product length (bp)
bta-miR-21	GCTAGCTTATCAGACTGATGTTGACTAAA	29
bta-miR-146a-5p F	TGAGAACTGAATTCCATAGGTTG	23
GMS hsa-miR-155-5p	TGCTAATCGTGATAGGGGTAAA	22
bta-miR-34c-5p	AATCACTAACCACACGGCCAGG	22
RNU6	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT	42

Table III-1. Specific primer sequences for miRNAs

4. RESULTS

4.1. Effects of cytokine treatment on miRNA expression in cumulus cells

RT-qPCR quantification of miRNA transcripts in RNA isolated from bovine cumulus cells is shown in Figure 1. MiR-21 transcript levels rose significantly ($P<0.05$) in cumulus cells in the control group and after the LIF treatment when compared to the cumulus cells from COCs at the germinal vesicle stage MiR-155 expression significantly ($P<0.05$) increased under standard maturation conditions, and no signficant change was found in the other treatment groups. There was no change in expression for miR-146a and miR-34c.

4.2. Effects of cytokine treatment on miRNA expression on oocytes

The expression pattern and cytokine responsiveness of miRNAs examined in oocytes (Figure 2) was slightly different from that observed in cumulus cells. The expression of miR-21 was significantly ($P<0.05$) increased when oocytes were treated with LIF, whereas that of miR-146a was significantly when IL-11 was added to the maturation medium. In contrast, the addition of IL-6 had no impact on the expression levels of miR-21 and miR-146a. Expression levels of miR-34c and miR-155 were not affected by LIF, IL-6 or LIF-11.

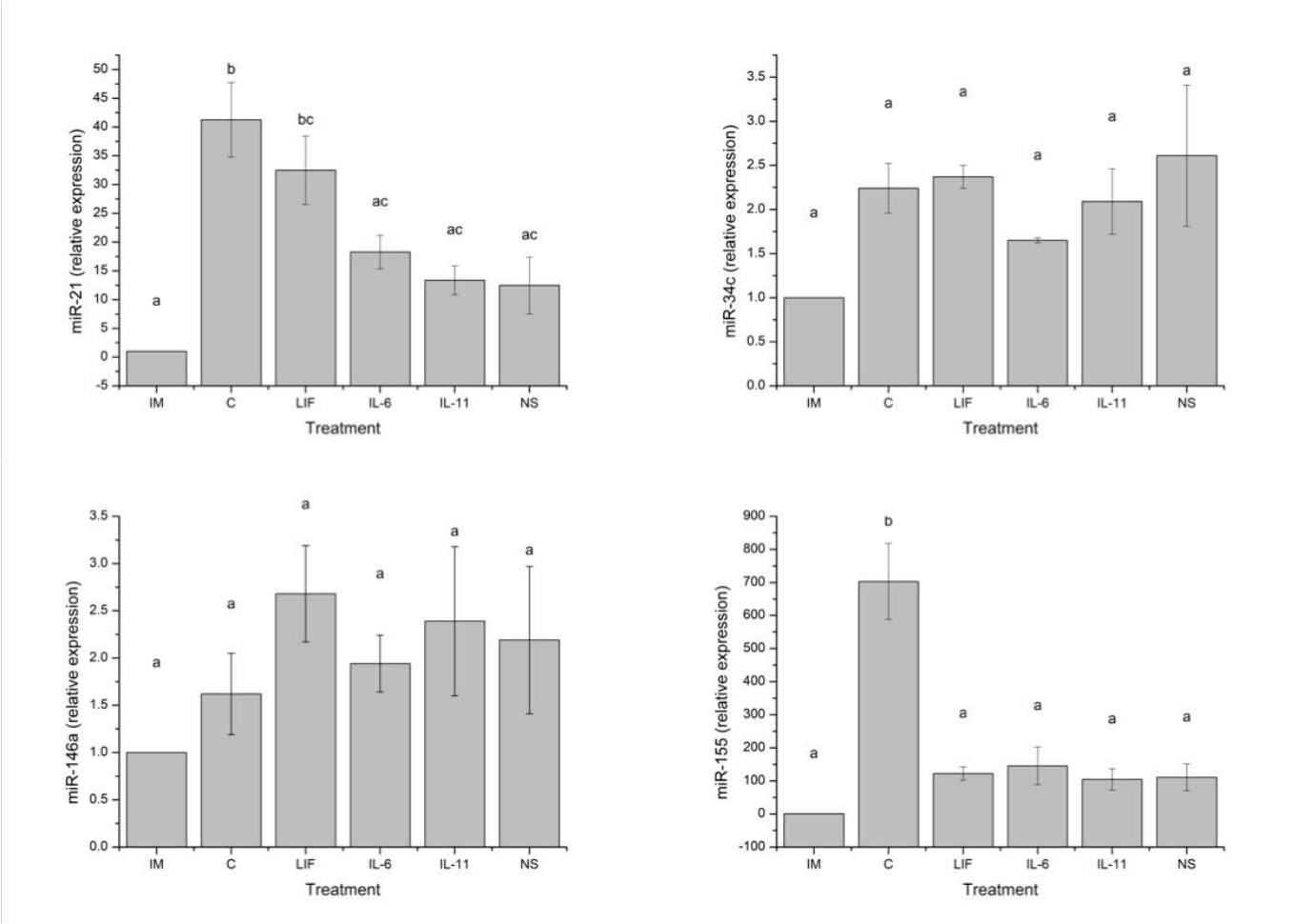


Figure III-1. RT-qPCR quantification of miRNA transcripts in bovine cumulus cells from maturing COCs. COCs were randomly distributed into 6 groups, corresponding to: IM: immature oocytes or germinal vesicle; C: control; LIF: treated with 25 ng/mL LIF; IL-6: treated with 10 ng/mL IL-6; IL-11: treated with 5 ng/mL IL-11; and NS: non-supplemented.

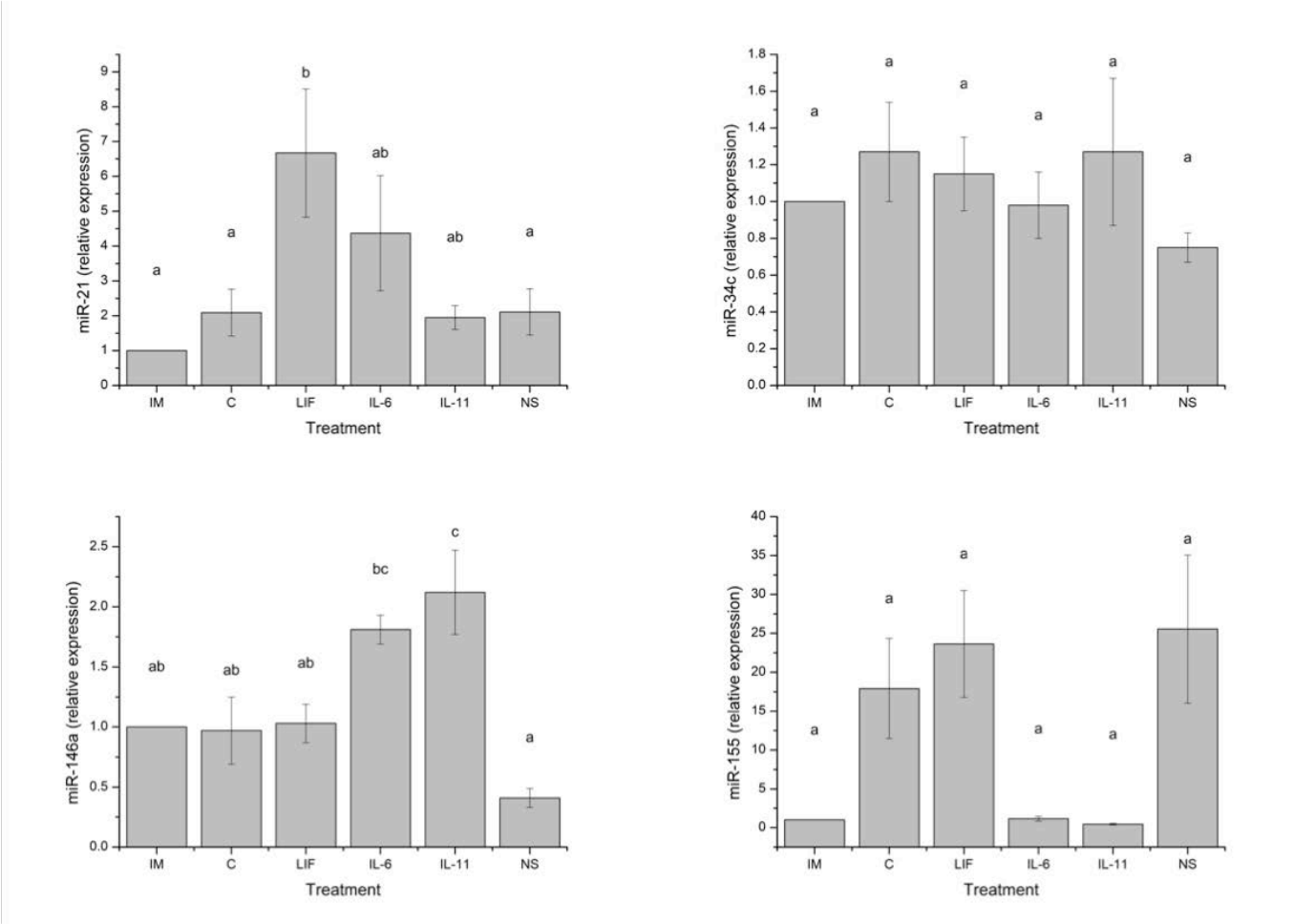


Figure III-2. RT-qPCR quantification of miRNA transcripts from bovine oocytes. COCs were randomly distributed in 6 groups, corresponding to: IM: immature oocytes or germinal vesicle; C: control; LIF: treated with 25 ng/mL LIF; IL-6: treated with 10 ng/mL IL-6; IL-11: treated with 5 ng/mL IL-11; and NS: non-supplemented;

5. DISCUSSION

Factors that regulate the expression of miRNAs in the context of the oocyte and ovarian follicle have not been widely studied. In the present study, COCs treated with LIF showed higher expression of miR-21 in both cumulus cells and oocytes, an expected result observed in previous studies [160] . The promoter region of miR-21 contains two binding sites for STAT3. STAT3 is a signal transducer activated by LIF through its receptor subunits, GP130 and LIFR, upon LIF engagement. miR-21 has been described, in different species, as the most abundant miRNA in cumulus cells [179], [182] and it is present throughout folliculogenesis [182] . Some studies have proposed that miR-21 should be considered a potential biomarker for oocyte quality, as it has been reported that increased miR-21 expression is associated with a reduction in apoptosis (X. Han et al. 2017). Surprisingly, despite the fact that IL-6 activates STAT3 phosphorylation in myeloma cells [185] , we did not observe any significant increase in miR-21 with other cytokines examined in either cumulus cells or oocytes.

MiR-146a has been detected both in immature and mature cumulus cells and oocytes, and it has been described to decrease dramatically during oocyte maturation [184]. However, this reduction was not observed in our studies, nor were changes induced by LIF, IL-6 or IL-11 with respect to the expression of miR-146a in cumulus cells. However, IL-11 significantly increased miR-146a levels in oocytes. Our results of miR-146a on oocytes may be due to activation of pathways other than STAT3, as characterized by Li et al. (2012) [186]. Their study in human chondrosarcoma cells first characterized the IL-11 signaling pathway that leads to an activation of NF-κB, a transcription factor associated with IL-6 expression (Sheldon and Bromfield 2011). Hexameric complexes involving IL-11, IL-11R, and GP130 (Matadeen et al. 2007) activate the PI3K/AKT pathway via JAK. AKT phosphorylates IKK, which in turn targets IκB for ubiquitination, releasing NF-κB, which then translocates into the nucleus and interacts with the promoter region inducing miR-146a expression. This activation of an alternative signaling pathway may help explain the selective effect of IL-11 on miR-146a in the present context.

LIF, IL-6 or IL-11 did not affect the expression of miR-34c a key member of the larger family members (34a,b,c) important for gametogenesis and embryo development [186]–[189]. miR-34c has been detected in bovine germinal vesicle, mature oocytes and 2-cell embryos [190] . In somatic cells, the p53 tumor suppressor is involved in regulating miR-34 expression, inducing cell cycle arrest [191], [192] . Activation of p53 induces miR-34c expression, leading to an inhibition of BCL2 expression, driving cells to apoptosis [192] . This process has also been reported in mouse oocytes [186] . It is therefore perhaps not surprising LIF, IL-6 or IL-11 did not induce the expression of miR-34c, as this would likely be more reflective of cellular stress.

In the present study, miR-155 expression was increased in control cumulus cells, whereas no significant differences were observed in cumulus cells from COCs treated with LIF, IL-6 or IL-11. A consistent, but non-significant trend towards increased levels of miR-155 was observed in oocytes from LIF and control groups in addition to those undergoing normal in vitro maturation. Detectable levels of miR-155 have been reported both in bovine immature and mature cumulus and oocytes [184] , and it is one of the most abundant miRNAs in bovine cumulus cells [179] . Functionally, miR-155 is one of the most prominent miRNAs in inflammatory processes, and it is associated with ROS induction through the suppression of antioxidant genes [193] . After induction through TLR4 (toll-like receptor 4) miR-155 [194] and miR-146a [195], [196] act as negative feedback regulators of inflammation, inhibiting TAB2 (TGF-beta activated kinase 1 (MAP3K7) binding protein 2) [197] a kinase in the NF-κB pathway. Thus, miR-146a and miR-155 exert anti-inflammatory properties by down-regulating IL-6 and other target genes. In the present context our data suggest that levels of miR-155 tend to be lower when IL-6 and IL-11 are added to the maturation medium. Given the overlap between inflammation and follicular events, the anti-inflammatory role of miR-155 could thereby provide one potential explanation for the elevated levels in untreated cumulus cells, and in control and LIF-treated oocytes where such feedback has not occurred.

Chapter IV

Effect of the addition of LIF during *in vitro* maturation on embryo development and gene expression

1. ABSTRACT

Maternal effect genes play a crucial role in early embryogenesis. *In vitro* culture conditions may affect maternal gene expression in bovine oocytes and embryos. We investigated whether *in vitro* maturation medium supplementation with LIF (leukemia inhibitory factor) affects the mRNA levels of bovine MII oocytes and embryos. Cumulus–oocyte complexes (COCs) were matured in maturation medium (TCM199 supplemented with 20% FCS and EGF (10 ng/mL and gentamicin) (Control) or in maturation medium supplemented with LIF (25 ng/mL). After maturation for 24h, COCs were in vitro fertilized. In a first experiment, germinal vesicle oocytes, MII oocytes, 2- and 8-cell embryos were preserved for the analysis of mRNA levels of ten candidate genes (*ZAR1*; *NPM2*; *DPPA3*; *DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*). In a second experiment, cleavage rates and blastocyst rates were assessed and day 8 blastocysts were harvested for the analysis of mRNA levels of seven candidate genes (*DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*). No changes in expression of *ZAR1*, *NPM2*, *DPPA3*, *DNMT3A*, *KAT2A*, *HDAC1*, *BAX*, *BCL2L1* and *BAX*:*BCL2L1* ratio were observed for MII, 2- and 8-cell stage embryos. However, maturation with LIF significantly ($P<0.05$) increased the expression of *HSPA1A* in 2-cell stage embryos or the expression of *HSP90AA1* in 8-cell stage embryos. No effects of LIF supplementation during maturation were observed when cleavage rate or blastocyst yield were assessed. When gene expression was analyzed on day 8 blastocysts, no changes in mRNA expression were observed for *KAT2A*, *HDAC1*, *HSP90AA1*, *BAX*, *BCL2L1* and *BAX*:*BCL2L1* ratio. However, expanded and hatched embryos from oocytes *in vitro* matured with LIF showed a significant decrease ($P<0.05$) for DNMT3A-transcript levels while HSPA1A-transcript levels were significantly ($P<0.05$) lower in hatching and hatched blastocysts produced from LIF-matured oocytes. The inclusion of LIF in the bovine in vitro maturation system influences mRNA expression of two stress-inducible heat-shock proteins (*HSPA1A* and *HSP90AA1*) in 4 or 8-cell embryos while it decreases mRNA expression of *DNMT3A* and *HSPA1A* in bovine blastocysts in vitro.

2. INTRODUCTION

Abattoirs are a common and cheap source of ovaries intended for research. It is, though, a heterogeneous source of oocytes in terms of origin (maternal genetics merit, nutrition and age, inflammatory diseases, environmental contaminants and thermal stress) what ultimately defines oocyte quality. Of the total collected oocytes, around 90% of immature oocytes undergo nuclear and cytoplasmic maturation reaching metaphase II. Approximately 80% are fertilized and develop until 2-cell stage. It is common, though, that just the half of them endures the whole developmental process until day 7 or 8. At this point, it is logical to think in vitro culture (IVC) environment is the major responsible of this depletion. However, there are evidences that the quality of the oocyte is emphasized (highlighted) as the limiting factor (reviewed by [198], [199]). In vitro procedures still need to be improved, as the results obtained still differ from the in vivo ones [98], [99] . When oocytes undergo maturation in vitro, blastocyst yield is usually limited around 35%, still when they are matured in vivo the rate advances up to 80%, independently IVC was in vitro or in vivo (reviewed by [198]). This results get even more compromised when oocytes go through cryopreservation procedures.

Interleukin (IL) -6 family of cytokines is characterized to share a common signal transducer receptor chain, the glycoprotein 130 (GP130). This trait brings together ten members: leukemia inhibitory factor (LIF), IL-6, IL-11, IL-27, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), cardiotrophin-like cytokine factor 1 (CLCF1), IL-35 and IL-39. IL-6 family members play integral roles in numerous physiologic and pathophysiologic processes. Their pleiotropic members are engaged in homeostasis, immunity, infection and inflammation. Their functions are partially overlapping and redundant, but they can also be involved in well-defined and singular actions. Formation of cytokine signaling complexes on the cellular membrane triggers activation of the intracellular Janus kinase, initiating a cascade that activates three different pathways: JAK/STAT, MAP-kinase and PI(3)-kinase (reviewed by [105]–[108]).

LIF has been broadly studied around its role in the reproductive function. Several studies have been carried out on the use of LIF as a supplement to the maturation media in mouse and livestock species. It has been reported to promote oocyte maturation in mice [141] , bovine [142] , pig[143] and slightly in goat [144] , and its effects persisted until early developmental stages, even improving the blastocyst rate [141], [142] . When supplemented in the culture media it improved cleavage rate in sheep [145], [146], [169] , early developmental stages [147], [148] and developmental competence in mice [149] . In terms of blastocyst quality, LIF treated embryos had better hatchability [148], [150] , and embryos were described to increase in total cell number in mouse [149] , bovine[142], [151] , buffalo [150] and sheep, also presenting a grater proportion of inner cell mass number [146] . LIF has also been described as an important factor for blastocyst implantation [152], [153] , trophoectoderm proliferation [154] and invasion [155] .

It seems clear then, looking for changes on maturation environment could improve the final blastocyst yield. The microenvironment provided to the oocytes will affect both oocyte and embryo gene expression [100], [101] .

There is a certain consensus in which transcription in oocytes is considerably reduced beyond the germinal vesicle break down. Consequently, oocyte relies on maternal transcripts that have been stored during oogenesis [200]. Maternal-to-embryonic (MET) transition is the period when the control of development is transferred from the maternal to the zygotic genome. As early development advances, maternal derived transcripts and proteins are degraded, whereas embryonic genome is activated (reviewed by [201], [202]). The time at which embryonic genome activation (EGA) occurs varies between species, and it seems to take place at 8- to 16-cell stage in bovine [200] . EGA occurs in different waves, during the early steps in development. Timing of cleavage, EGA itself, compaction and blastocyst formation can be affected by the close environment, so analyzing gene expression during early development seems to be a good time point to evaluate our treatment. Maternal effect genes (MEG) are expressed in oocytes and embryos and play important roles in EGA. Assessing their transcript levels as well as other genes associated to blastocyst quality, inferring epigenetics, apoptosis and

stress, could help us understand the effect of changing the milieu during maturation (reviewed by [201], [203], [204]). Therefore, the aim of this study is to evaluate the effect of LIF supplementation during bovine oocyte maturation on embryo development and gene expression.

3. MATERIAL AND METHODS

3.1. Chemicals and suppliers

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated.

3.2. Oocyte collection and *in vitro* maturation

The in vitro protocols followed for maturation (IVM), fertilization (IVF) and culture (IVC) have been described previously by Rizos et al (2001). Ovaries from heifers (>12 months of age) and cows (>24 months of age) were transported from a local slaughterhouse in phosphate-buffered saline (PBS) at 35–37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2–10 mm follicles. Only COCs with three or more layers of cumulus cells and a homogeneous cytoplasm were selected for in vitro maturation. After three washes in modified Dulbecco's PBS (PBS supplemented with 0.036 mg/mL pyruvate, 0.05 mg/mL gentamicin and 0.5 mg/ml bovine serum albumin (BSA)), groups of up to 20 COCs were placed in 100 µL drops of maturation medium and cultured for 24 h at 38.5ºC in a 5% CO2 humidified air atmosphere. The maturation medium consisted of TCM-199 supplemented with 10% (v/v) FBS, 10 ng/mL epidermal growth factor and 50 µg/mL gentamicin. LIF treated oocytes were matured with the same IVM medium additionally supplemented with human recombinant LIF (25 ng/mL). Immature oocytes in germinal vesicle stage (GV) and mature oocytes (MII) were collected for RNA extraction, reverse transcription and qPCR analysis.

3.3. *In vitro* fertilization and embryo culture

In vitro matured oocytes were in vitro fertilized at 38.5ºC in a 5% CO2 atmosphere. Briefly, frozen/thawed spermatozoa from Asturian bulls (ASEAVA,

Llanera, Asturias, Spain) of proven fertility were used in all the experimental procedures. High motility and good morphology spermatozoa were obtained by centrifuging frozen/thawed sperm at 300 x g and room temperature for 10 min on a discontinuous gradient composed by 1 mL of 40% and 1 mL of 80% BoviPure (Nidacon Laboratories AB, Göteborg, Sweden) according to the manufacturer's specification. Viable spermatozoa collected from the bottom were washed with 3 mL of BoviWash (Nidacon International, Göteborg, Sweden) and pelleted by centrifugation at 300 x g for 5 min. Spermatozoa were counted in a Neubauer chamber and diluted in an appropriate volume of fertilization medium (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 1 mg/mL heparin-sodium salt) to a final concentration of 1 x 106 spermatozoa/mL. 100 µL droplets of diluted sperm were made under mineral oil and 20 oocytes/droplet were co-incubated at 38.5ºC, 5% CO2 and high humidity.

After 18–20 h, the presumptive zygotes were stripped of remaining cumulus cells by pipetting and cultured in groups of 20 in 20 µL drops of IVC medium, consisting of Synthetic Oviduct Fluid (Caisson Labs, Smithfield, USA) supplemented with 0.96 µg/mL BSA, 88.6 µg/mL Na-pyruvate, 2% non-essential amino acids, 1% essential amino acids, 0.5% gentamicin and 2% FBS under mineral oil. Presumptive zygotes were incubated at 38°C in a humidified 5% CO2 and 5% O2 atmosphere for 8 days.

Embryo development was recorded on day 2 (cleavage), and days 7 and 8 (blastocysts) post-insemination (pi). Day 8 embryos were classified according to the degree of blastocoel expansion into three groups based on Manual of the International Embryo Transfer Society by Stringfellow & Seidel (1998): (1) non-expanded blastocysts: blastocoel volume less than one-half of the total volume of the blastocyst; (2) expanded blastocysts: blastocoel volume more than one-half of the total volume of the blastocyst; (3) hatching or hatched blastocysts: expanded blastocyst with an opened zona pellucida or lacking the zona pellucida. 2-cell (31-33 hpi), 8-cell (52-54 hpi) and day 8 blastocysts were harvested for RNA extraction, reverse transcription and qPCR analysis.

3.4. RNA extraction, reverse transcription and quantitative real-time PCR analysis

All the collected samples for gene expression analysis underwent the same RNA isolation protocol. Samples were washed three times with Dulbecco's PBS supplemented with 0.01% PVA at 38.5°C and then pipetted into 1.5 mL microtubes. Immediately, tubes were plunged into liquid nitrogen and stored at −80°C until further processed. GV and MII oocytes were pooled in groups of 20 and embryos in groups of 5. Poly-(A)-RNA was extracted using Dynabeads mRNA Direct Extraction Kit (Invitrogen™, Oslo, Norway), following the manufacturer's instructions with minor modifications. For poly-(A)-RNA extraction, pooled samples were lysed in 50 µL of lysis buffer for 5 min with gently pipetting, and the fluid lysate was then hybridized with 10 µL pre-washed beads for 5 min with gently shaking. After hybridization, poly-(A)-RNA-bead complexes were washed twice in 50 µL of Washing Buffer A and two more times in 50 µL of Washing Buffer B. Next, the samples were eluted in 16 µL of Elution Buffer (Tris HCl) and heated to 70°C for 5 min. Immediately after extraction, 4 µL of qScript cDNAsupermix (Quanta Biosciences; Gaithersburg, MD, USA) were added and the reverse transcription (RT) reaction was carried out using oligo-dT primers, random primers, dNTPs and qScript reverse transcriptase. The RT reaction was performed for 5 min at 25°C, followed by 1 h at 42°C to allow the RT-PCR of mRNA and 10 min at 70°C to denature the reverse transcriptase enzyme. After RT, the cDNA was diluted with 25 µL of Tris HCl (elution solution).

Quantification of relative abundance of mRNA transcripts was performed by the qPCR method using a 7500 Real Time PCR System (Applied Biosystems, Foster City, California, USA). The qPCR reaction mix contained 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA), 1 µL of forward and 1 µL of reverse primers (Life Technologies, Madrid, Spain) and 2 µL of cDNA template. Nuclease-free water was added up to a final volume of 20 µL. The PCR amplification was carried out for one cycle of denaturation at 95°C for 10 min, 45 cycles of amplification with denaturation step at 95°C for 15 seconds, annealing step for 1 min at 60ºC (the appropriate annealing temperature of primers) and

extension step at 72°C for 40 seconds. Fluorescence data were acquired during the 72°C extension steps. To verify the identity of the amplified PCR product, melting curve analysis and gel electrophoresis (in a 2% agarose gel containing 0.6 μg/mL ethidium bromide) were performed. The melting protocol consisted of heating the samples from 50 to 95°C, holding at each temperature for 5 seconds, while monitoring fluorescence. In each run there were three technical replicates from each of the three biological replicates per individual gene. Furthermore, negative controls for the template and for the reverse transcription were also included and amplified by PCR to ensure that no cross-contamination occurred.

Seven candidate genes (DNMT3A; KAT2A; HDAC1; BAX; BCL2L1; HSPA1A; HSP90AA1) for blastocysts and ten (ZAR1; NPM2; DPPA3; DNMT3A; KAT2A; HDAC1; BAX; BCL2L1; HSPA1A; HSP90AA1) for GV, MII, 2-cell and 8-cell embryos were used to perform quantitative PCR analysis in comparison with glyceraldehide-3-phosphate dehydrogenase (GAPDH) and H2A histone family member Z (H2AFZ) genes (endogenous control genes). The comparative threshold cycle (Ct) method was used to quantify relative gene expression levels and quantification was normalized to the endogenous control (housekeeping (HK) genes: GADPH and H2AFZ. Fluorescence data were acquired after each elongation step to determine the threshold cycle for each sample. The threshold cycle, which is set in the log-linear phase, reflects the PCR cycle number at which the fluorescence generated within a given reaction is just above background fluorescence. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative Ct method, the ΔCt value was determined by subtracting the mean between GAPDH and H2AFZ Ct values for each sample from the Ct value of each target gene of the sample for each replicate separately. Calculation of ΔΔCt involved the subtraction of the ΔCt value for the untreated (GV in experiment 1 and early control fresh embryos for experiment 2) from all the other ΔCt sample values. Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-(\Delta\Delta Ct)}$, the Livak and Schmittgen method. Primer sequences, amplicon size and GenBank accession numbers for each gene are provided in Table 1. The efficiency of primer

amplification was 100%. Non-template controls were not amplified or returned a Ct value 10 points higher than the average Ct value for the genes. The experiment was repeated independently three times.

3.5. Statistical analysis

Statistical tests were performed using the statistical package IBM SPSS Version 25.0 for Windows (IBM Corp.; Armonk, NY, USA). Cleavage rates, blastocyst yields and relative transcript abundances were evaluated by a one-way analysis of the variance (ANOVA). Data are expressed as means ± standard error of the mean (SEM). Significance was set at $P \leq 0.05$.

3.6. Experimental design

EXPERIMENT 1. Characterize the effects of LIF added to the maturation media on specific gene expression on oocytes and early embryos.

Upon oocyte collection, oocytes were randomly allocated in three groups: GV, control and LIF. GV oocytes were collected and immediately flash frozen for RT-qPCR analysis. Oocytes from control and LIF groups were in vitro matured. At 24h of IVM, groups of 20 MII oocytes from control and LIF groups were collected, and the rest were fertilized and cultured. Embryo development was allowed to continue until 31-33 hpi and 52-54 hpi, moment in which 2- and 8-cell embryos respectively were harvested.

EXPERIMENT 2. Characterize the effect of LIF added to the maturation media on developmental competence and specific gene expression. After 24h of IVM, oocytes from both groups were in vitro fertilized and cultured. Cleavage rates were determined at 48 hpi and blastocyst rates on day 7 and day 8 pi (four replicates per group). At day 8, embryos were harvested for RNA extraction and RT-qPCR.

Symbol	Gene name	NCBI Reference Sequence	Primer set sequences (5'-3')	Length (nt)
BAX	BCL2 associated X, apoptosis regulator	NM_173894.1	F: GAGAGGTCTTTTTCCGAGTGGC R: TGTCCCAAAGTAGGAGAGGAG	237
BCL2L1	BCL2 like 1	BC147863.1	F: CCACTTAGGACCCACTTCTGAC R: GGGTGCTTCCTACAGCTACAGT	188
DNMT3A	DNA methyltransferase 3 alpha	NM_001206502.1	F: CCTCAGCTCCCCCTACTTATTC R: AGCTGTGAGCTTACTCCTGAGC	199
DPPA3	Developmental pluripotency associated 3	NM_001111108.2	F: TGGCTACTCTTCATCCCCTACA R: TCTAGGGTCCAGGTTGGGTT	230
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034.2	F: AGTCCACTGGGGTCTTCACTAC R: CAGTGGTCATAAGTCCCTCCAC	243
HDAC1	Histone deacetylase 1	NM_001037444.2	F: CTGAGGAGATGACCAAGTACC R: CCACCAGTAGACAGCTGACAGA	167
HSPA1A	Heat shock protein family A (Hsp70) member 1A	NM_203322.3	F: GCAGGTGTGTAACCCCATCA R: CAGGGCAAGACCAAAGTCCA	181
HSP90AA1	Heat shock protein 90 alpha family class A member 1	NM_001012670.2	F: GTGGAGACTTTCGCCTTCCA R: TGGTGAGGGTTCGATCTTGC	223
H2AFZ	H2A histone family, member Z	NM_174809.2	F: GCGTATTACCCCTCGTCACTTG R: GTCCACTGGAATCACCAACACTG	227
KAT2A	Lysine acetyltransferase 2A	XM_015468132.1	F: AGGATGTGGCTACCTACAAGG R: GCACCAGCTTGTCCTTCTCTAC	190
NPM2	Nucleophosmin / Nucleoplasmin 2	NM_001168706.1	F: GGACCTGTGTTCCTCTGTGG R: CTTCACTTGTTTGACGGGCG	153
ZAR1	Zygote arrest 1	NM_001076203.1	F: GGGAGATGCAAAGGCAAACG R: CCAAACAACAGCCTTCCACG	216

Table IV-1. Primers used for reverse transcription–quantitative polymerase chain reaction.

4. RESULTS

4.1. Experiment 1. Effect of LIF supplementation during IVM on gene expression

RT-qPCR quantification of maternal effect genes transcripts is shown in Figure 1. Neither zygote arrest 1 (ZAR1), nucleoplasmin 2 (NPM2) nor developmental pluripotency associated 3 (DPPA3) presented changes in expression for MII, 2- and 8-cell stage embryos. Also, no difference in expression was found for genes involved in epigenetics (DNMT3A, KAT2A and HDAC1; Figure 2) and apoptosis (BAX, BCL2L1, BAX:BCL2L1 ratio; Figure 3). However, a tendency could be appreciated for DNMT3A ($P=0.078$) and HDAC1 ($P=0.091$) on 8-cell stage embryos derived from oocytes that had been matured with LIF.

HSPA1A expression significantly ($P<0.05$) increased in 2-cell stage embryos, those derived from LIF-treated oocytes. Under the same treatment, at 8-cell stage, a similar trend could be recognized ($P=0.097$) for HSPA1A. LIF rose also transcript levels for HSP90AA1 in 8-cell stage embryos, whereas those of MII and 2-cell zygotes remained similar between treatments.

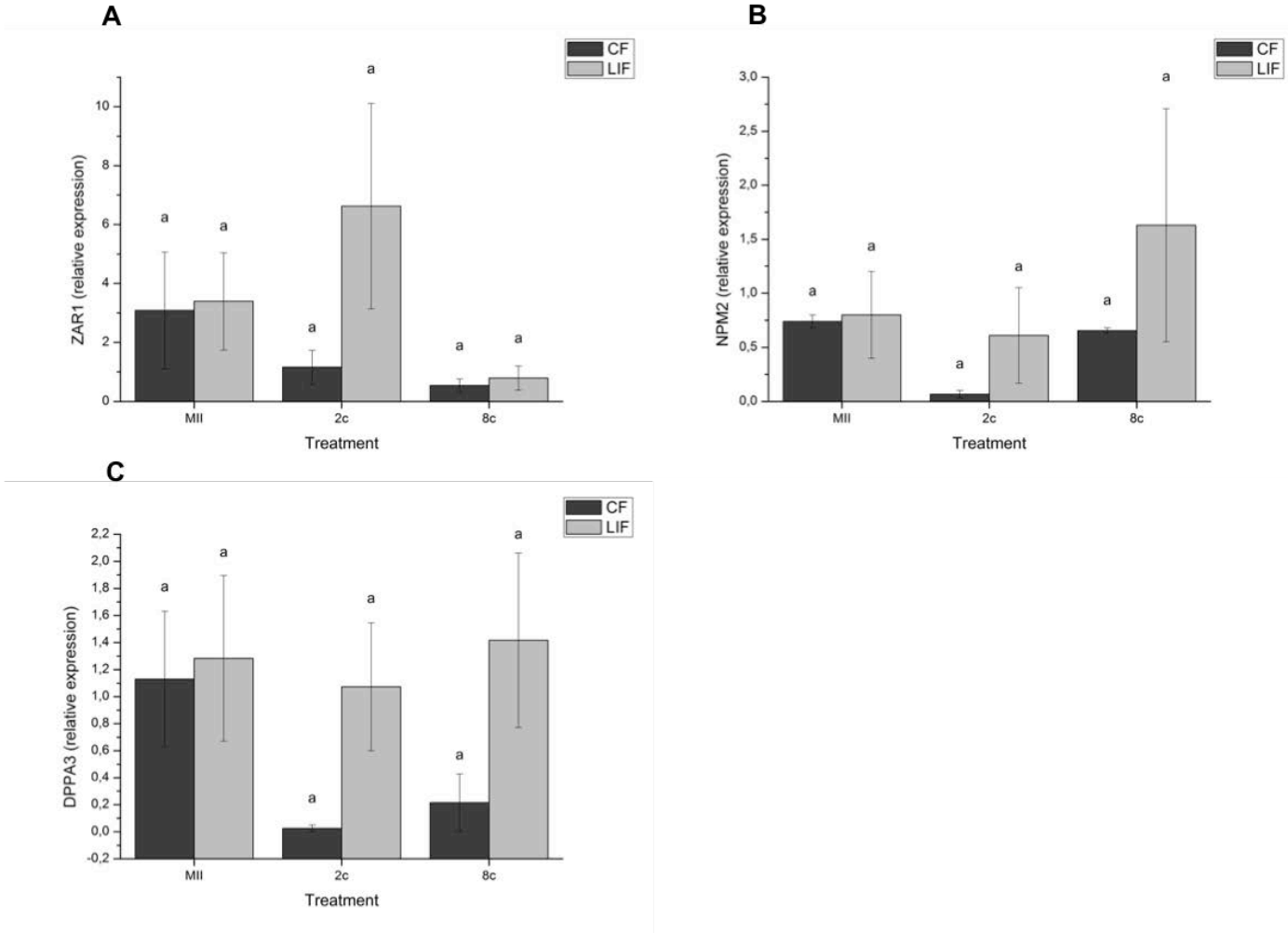


Figure IV-1. Relative abundance (as mean ± SEM) of maternal effect genes. (A) ZAR1, (B) NPM2 and (C) DPPA3 in MII oocytes, 2- and 8-cell embryos from control and LIF-treated groups. Different superscripts indicate significant differences (P<0.05).

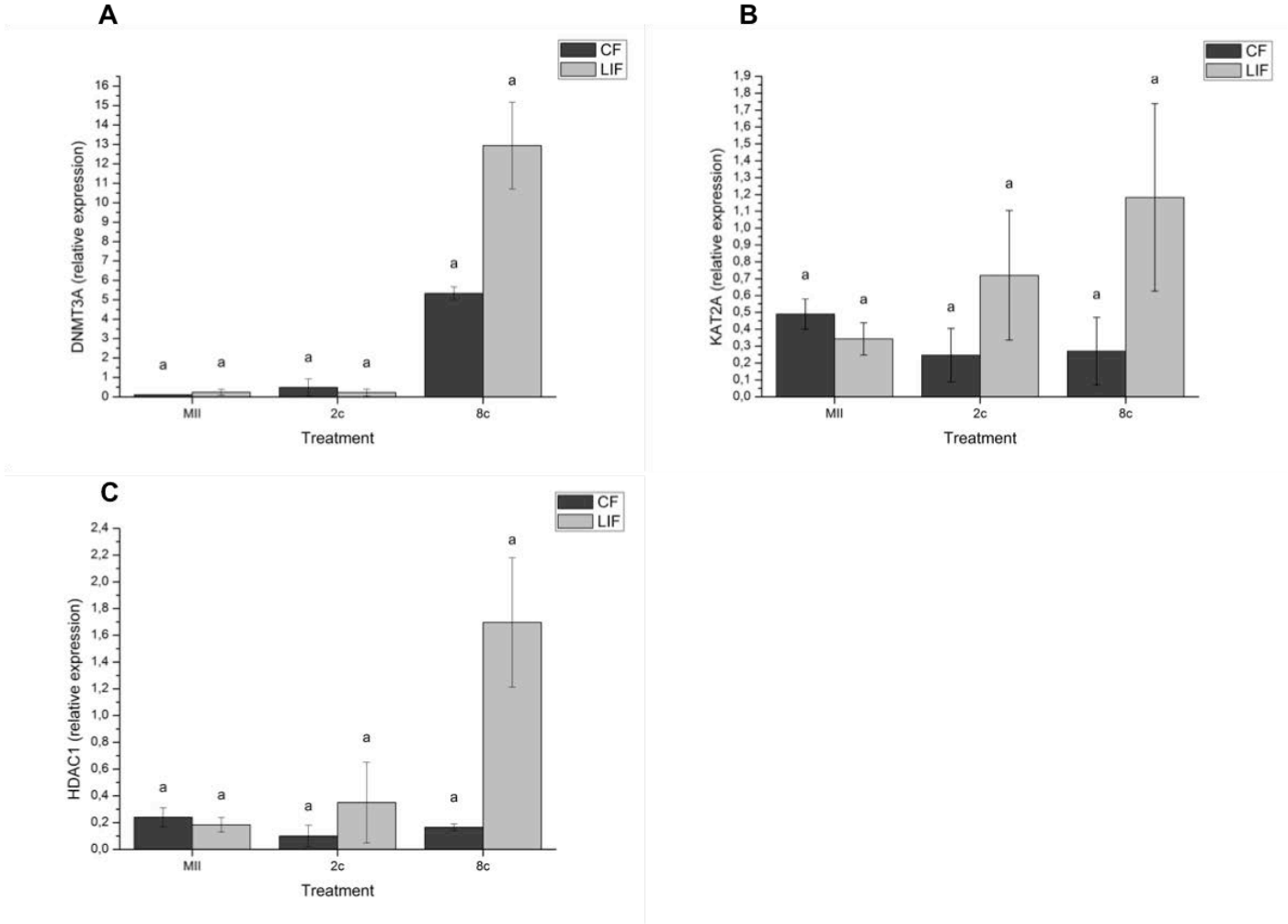


Figure IV-2. Relative abundance (as mean ± SEM) of genes involved in epigenetic reprogramming. (A) *DNMT3A*, (B) *KAT2A* and (C) *HDAC1* in MII oocytes, 2- and 8-cell embryos from control and LIF-treated groups. Different superscripts indicate significant differences (P<0.05).

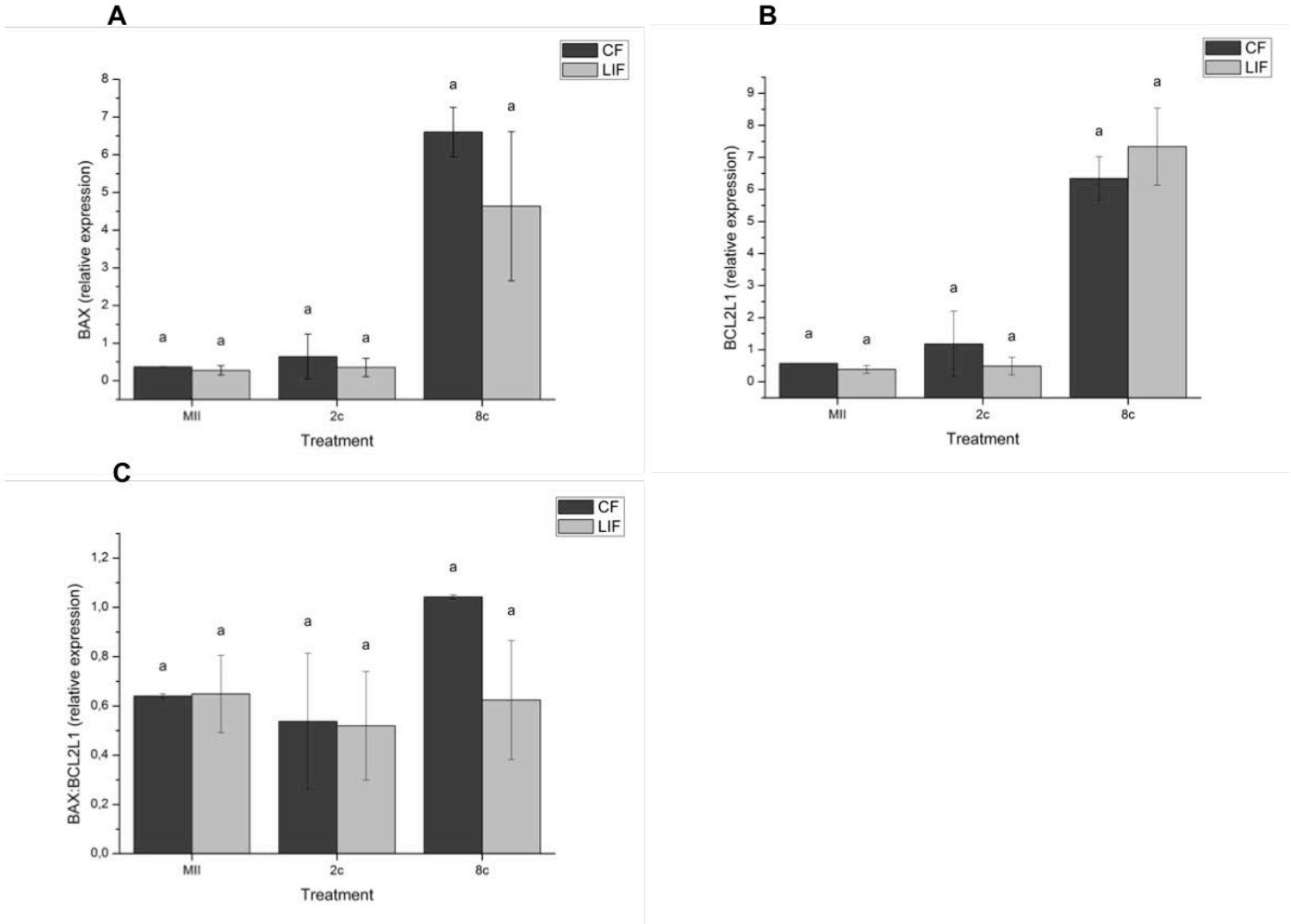


Figure IV-3. Relative abundance (as mean ± SEM) of genes involved in apoptosis. (A) *BAX*, (B) *BCL2L1* and (C) *BAX:BCL2L1* ratio in MII oocytes, 2- and 8-cell embryos from control and LIF-treated groups. Different superscripts indicate significant differences (P<0.05).

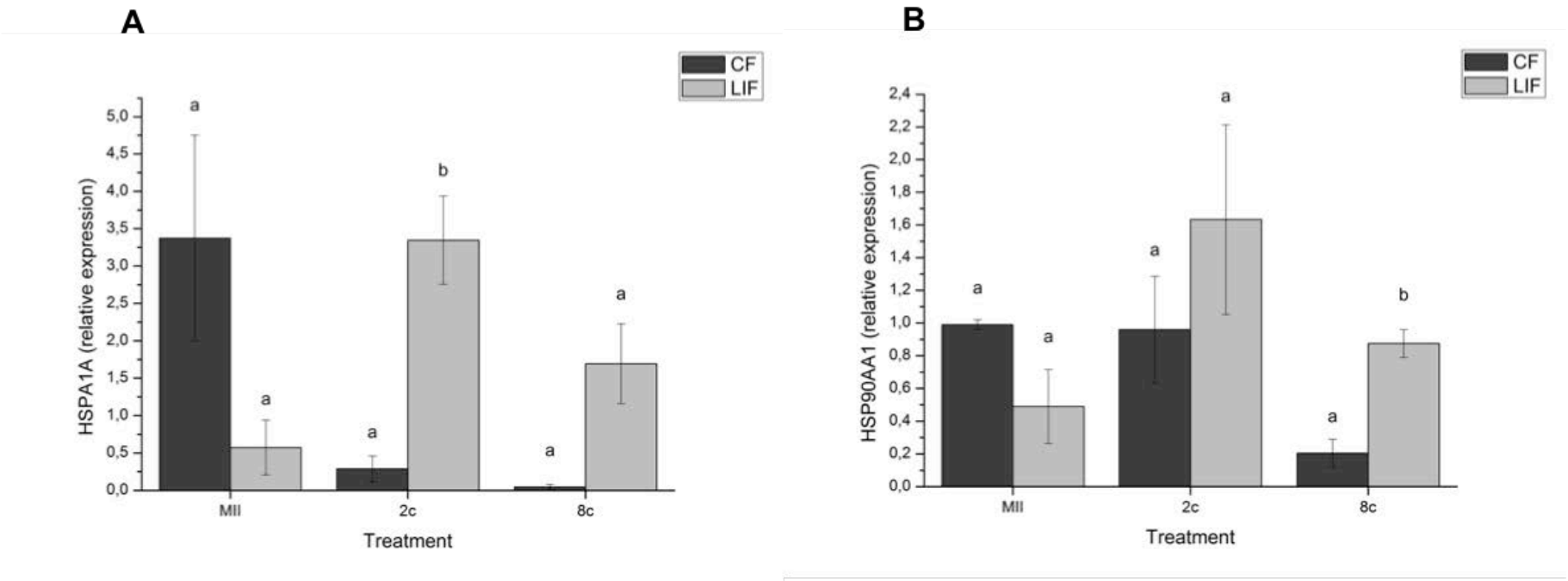


Figure IV-4. Relative abundance (as mean ± SEM) of genes involved in heat stress. (A) *HSPA1A* and (B) *HSP90AA1* in MII oocytes, 2- and 8-cell embryos from control and LIF-treated groups. Different superscripts indicate significant differences ($P<0.05$).

4.2. Experiment 2. Effect of LIF supplementation during IVM in blastocyst outcome and gene expression

When the effects of LIF supplementation during maturation on embryo development were assessed (Table 2), no significant differences were observed in relation to cleavage rate. Additionally, no changes in yield were found neither on day 7 and day 8 blastocyst, nor in terms of hatchability.

In relation to gene expression, LIF treated embryos at expanded and hatched stage showed a significant decrease ($P<0.05$) for DNMT3A-transcript levels (Figure 5), while no difference was found in other stages. No differences were appreciated for KAT2A and HDAC1. The same results in relation to significance were observed for the pro-apoptotic BAX gene, the anti-apoptotic BCL2L1, as well as the ratio between them.

Genes related to heat stress presented a different expression pattern when compared to the expression on MII, 2- and 8-cell zygotes. HSPA1A-transcript levels were significantly ($P<0.05$) lower under LIF treatment in hatching and hatched blastocysts, while no other group showed any relevant change. The expression levels of HSP90AA1 did not show any modification along embryo development.

					D8 Blastocyst			
	n	**Cleavage**	**D7 Blastocyst**	**D8 Blastocyst**	**n_{D8}**	**Non-expanded**	**Expanded**	**Hatched**
Control	323	86.50±4.16[a]	24.64±4.13[a]	34.52±5.11[a]	116	15.86±3.92[a]	35.95±4.21[a]	48.19±6.59[a]
LIF	343	80.28±4.60[a]	24.42±4.49[a]	29.70±2.96[a]	102	6.99±3.53[a]	34.41±4.01[a]	58.60±5.36[a]

Table IV-2. Development of oocytes supplemented with 25 ng/mL of LIF during oocyte maturation. n = total of fertilized oocytes. Different superscripts indicate significant differences (P<0.05).

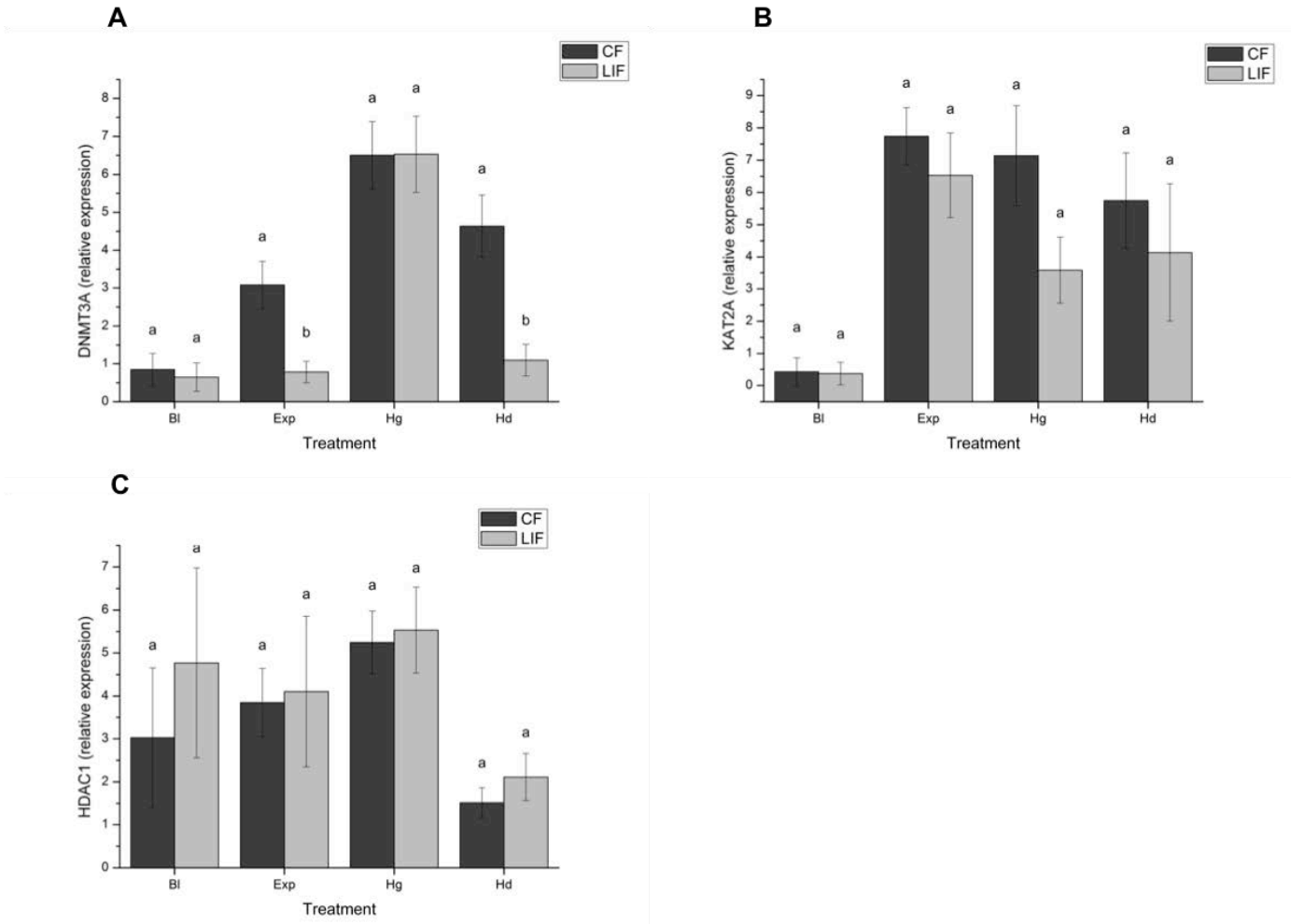


Figure IV-5. Relative abundance (as mean ± SEM) of genes involved in epigenetic reprogramming. (A) *DNMT3A*, (B) *KAT2A* and (C) *HDAC1* in different blastocyst stages. Bl, blastocyst; Exp, expanded; Hg, hatching; Hd, hatched. Different superscripts indicate significant differences (P<0.05).

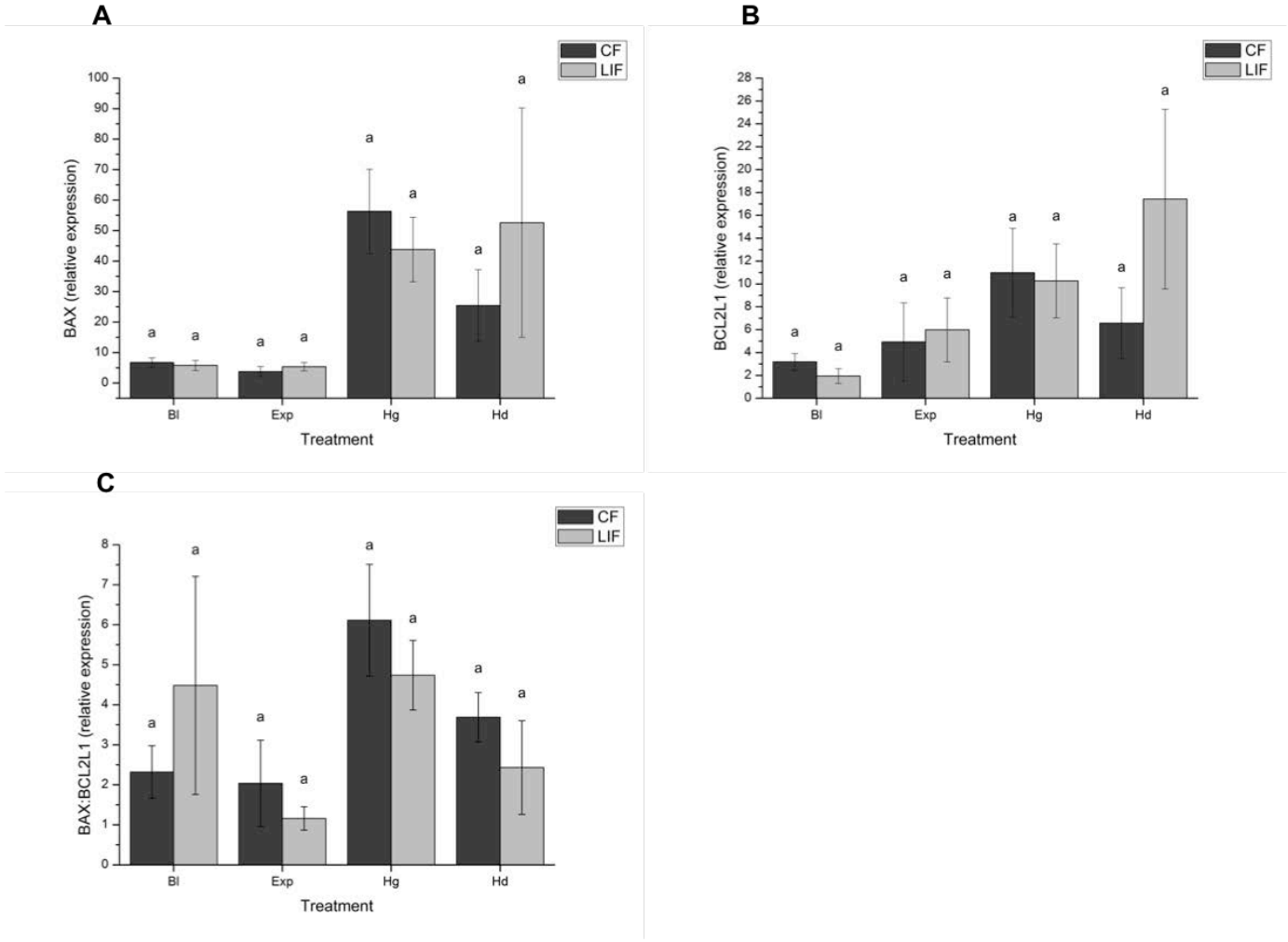


Figure IV-6. Relative abundance (as mean ± SEM) of genes involved in apoptosis. (A) *BAX*, (B) *BCL2L1* and (C) *BAX: BCL2L1* ratio in different blastocyst stages. Bl, blastocyst; Exp, expanded; Hg, hatching; Hd, hatched. Different superscripts indicate significant differences (P<0.05).

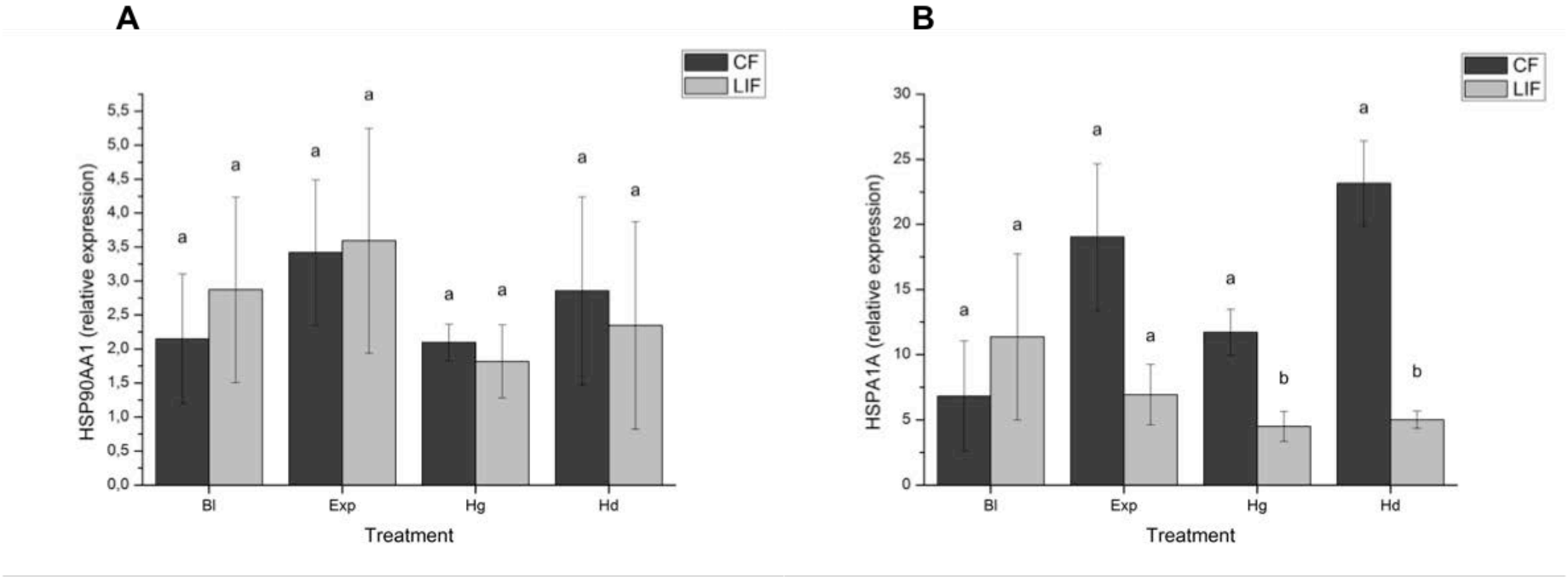


Figure IV-7. Relative abundance (as mean ± SEM) of genes involved in heat stress. (A) *HSP90AA1* and (B) *HSPA1A* in different blastocyst stages. Bl, blastocyst; Exp, expanded; Hg, hatching; Hd, hatched. Different superscripts indicate significant differences (P<0.05).

5. DISCUSSION

5.1. Experiment 1. Effect of LIF supplementation during IVM on gene expression

As stated previously, oocyte relies on maternal factors, products derived from maternal effect genes, before the transcription is resumed through the process called embryonic genome activation (EGA). Our results concerning *ZAR1*, *NPM2* and *DPPA3* did not present any significant differences between treatment groups. These data is supported by previous results in pig [205] were LIF supplementation during maturation did not induce any change in MII oocytes, 2- and 8-cell embryos. Even when LIF was added to the maturation media together with EGF and IL-1β no alteration was induced for *ZAR1* and *NPM2* but for *DPPA3,* the expression decreased in 8-cell stage [206] . Interestingly, this same study showed how MEG expression patterns differ when comparing *in vivo* and *in vitro* produced embryos. We expected different results at least for *DPPA3* on embryos derived from LIF-treated oocytes, as *DPPA3* is a direct target of the transcription factor STAT3, which is activated by LIF through its JAK/STAT signaling pathway (Wang *et al.*, 2018). After fertilization there is a generalized DNA demethylation of 5-methylcytosine content and *DPPA3* has been shown to protect the maternal material against it [207] . *DNMT1*, known as a maintenance methyltransferase, has been recently reported that could also work as a *de novo* methyltransferase. *DPPA3* protects oocyte genome from *de novo* methylation by *DNMT1*. This is a vital function, as an excessive DNA methylation impairs EGA, and so, embryo development [208] .

EGA occurs in different waves, the moment at which these transcription waves are given is also species-specific. In bovine, two waves are distinguished, a first and minor one around 2- to 4-cell stage embryos [201], [203] and a major or dominant one at 8- to 16-cell stage. The first wave activates transcription of genes involved in RNA processing, translation and transport, preparing the embryo for the major EGA, when genes involved in chromatin structure, transcription, RNA processing, protein biosynthesis, signal transduction, cell adhesion and

maintenance of pluripotency will be activated[201] . This is related to our findings, where we observed a tendency for increasing transcript levels on 8-cell treated embryos for *DNMT3A* and *HDAC1*. Methylation is a reversible epigenetic modification. *DNMT3A,* or DNA methyltransferase 3 alpha, is a *de novo* methyltransferase that methylates CpG dinucleotides. Promoter regions are usually rich in CpG and methylation induces gene silencing [209], [210] . Maturing oocytes with LIF seemed to increase transcription levels for *DNMT3A* at 8-cell stage compared to control, what according with a study performed by Lopera-Vasquez (2017) [211] is a sign of blastocyst quality. Bovine embryos cultured with oviductal fluid (OF) and those cultured with SOF supplemented with OF showed elevated levels of *DNMT3A*, total cell count and higher embryo cryotolerance, when compared with controls (SOF supplemented with serum). Similar results were obtained in our group when ooctyes were vitrified in the presence of the exopolysaccharide ID1 [14] . Lysine acetylation is another important epigenetic modification that modifies chromatin compaction. The steady-state level acetylated lysine residues in histones are controlled by histone acetyltransferases and histone deacetylases. Histone acetylation or deacetylation makes chromatin more or less accessible to the transcription machinery [212] . Our results did not reveal any relevant change in relative abundance for *KAT2A* (lysine acetyltransferase 2A). No changes in expression have been reported for *KAT2A* along embryo development in bovine [213] and mouse [214] and there is no data around *KAT2A* expression and LIF. Oocytes from diabetic mice showed increased levels for *KAT2A* and lower levels for *HDAC1* in mature oocytes [215] . 2-cell embryos produced from vitrified oocytes present elevated levels for *KAT2A*, considered a sign of oocyte impairment [149] . Our study showed a tendency at LIF-8-cell embryos for *HDAC1* (histone deacetylase 1). Transcript levels for that are not modified during oocyte maturation and early embryos until blastocyst stage [213] .

The addition of LIF into the maturation media did not produce any relevant modification in the derived embryos in terms of apoptosis. Neither *BAX*, *BCL2L1* nor the ratio between them showed any significant difference. In contrast, LIF has

been described to have an anti-apoptotic effect through the induction of microRNA-21 [216] .

It has been described previously that the stress inducible heat shock protein 70 kDa (*HSPA1A*) is spontaneously transcribed during the EGA [93] , which corresponds to the 8-cell stage in bovine. On the other hand, other studies revealed *HSPA1A*-transcript levels were higher at 2-cell stage compared to morulae and blastocyst [202], [217], [218]. Our data displayed an effect of LIF on 2-cell stage embryos, where *HSPA1A* was increased. During early embryo development, HSP70 could be a first system of protection and survival, since it has been attributed an anti-apoptotic role by binding to the tumor suppressor gene product p53 [219] . An increase of *LIF*-transcripts seems to be linked to an increase of *HSPA1A* [220] , and it is probably due to the activation of PI3K pathway. PI3K (phosphatidylinositol 3-kinase) activates AKT (AKT serine/threonine kinase 1) intensifying the expression of HSF1 (heat shock factor transcription 1), which in turn activates *HSPA1A* transcription [221] . In relation to *HSP90AA1*, our data demonstrated LIF could increase its expression on 8-cell derived zygotes. *HSP90AA1* has been studied in the context of cancer therapy for its role in apoptosis, which is diverse (reviewed by [222]). One of its anti-apoptotic functions is through interacting with AKT, which we have described previously as a member of PI(3)K pathway activated by LIF. AKT generates survival signal because of phosphorylation and consequent inactivation of pro-apoptotic proteins BAD and caspase 9 [223] . AKT also phosphorylates IkB, causing dissociation from NF-kB, allowing the transcription factor to translocate into the nucleus [224] . So, it is a good indicator that LIF could prompt *HSP90AA1* expression.

5.2. Experiment 2. Effect of LIF supplementation during IVM in blastocyst outcome and gene expression

As mention above, we did not observe any modification in terms of cleavage, blastocyst rate or hatchability when oocytes were submitted to a LIF treatment during maturation. These results are slightly contradictory with previous results from other works, were LIF seemed to increase the blastocyst yield (De Matos *et al.,* 2008; Mo *et al.*, 2014). When improved hatchability was observed in other

studies it was due to LIF administration during IVC [148], [150] , so maybe LIF effect could not persist long enough to make a difference in hatchability.

In relation to gene expression, the only changes in transcript levels observed were for *DNMT3A* and *HSPA1A*, where LIF induced a reduction in transcript relative abundance for those genes. *DNMT3A* significantly decreased in expanded and hatched blastocysts. In previous studies, elevated levels of *DNMT3A* in embryos produced from vitrified oocytes was associated to oocyte impairment [12], [14]. No modifications for the other genes involved in epigenetics were found, similar to the results found on MII oocytes, 2- and 8-cell embryos. Similar results appeared for *BAX*, *BCL2L1* and *BAX:BCL2L1* ratio. Different results were expected in apoptosis, as LIF has been described to induce *BCL2L1* through PI(3)K/AKT signaling pathway. Cardiac myocytes that had been induced apoptosis, this could be reversed thanks to the LIF treatment. LIF inhibits caspase 3 activation, allowing *BCL2L1* to recover basal levels [225] .

Hatching and hatched embryos exhibited lower levels of *HSPA1A*. Frezzato *et al.* [221] described two different models of how HSF1 is regulated by HSP70 protein. Elevated levels of HSP70 increased levels of active AKT that inhibits GSK3A/B by phosphorylation. This protein works as an inhibitor for HSF1, that now its inhibition leads to an upregulation of *HSPA1A*. When HSP70 is present in low levels, there is an upregulation of MAPK signaling pathway. Activated ERK can phosphorylate HSF1 causing inhibition and, therefore, preventing *HSPA1A* transcription.

Chapter V

Gene expression profiles and *in vitro* development following vitrification of bovine oocytes matured in media supplemented with LIF

1. ABSTRACT

An abnormality in gene expression profile in *in vitro* matured oocytes may lead to arrest of embryonic cleavage or to altered transcription of factors responsible for further embryonic development. Vitrified *in vitro* matured bovine oocytes have a lower developmental potential than fresh bovine oocytes. We hypothesized that *in vitro* maturation conditions and vitrification have an effect on gene expression at various embryos developmental stages, which may affect their developmental potential. Here, we examined the developmental capacity of vitrified/warmed bovine oocytes in vitro matured in a maturation medium supplemented with leukemia inhibitory factor (LIF). Further, we evaluated changes in mRNA profiles for ten genes (*ZAR1*; *NPM2*; *DPPA3; DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*) in GV, MII, 2-cell and 8-cell embryos and seven genes (*DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*) in day 8 blastocysts, using real-time polymerase chain reaction. No significant differences arouse neither in terms of cleavage rate nor blastocyst yield. No differences in the *ZAR1, NPM2, KAT2A, HDAC1, HSPA1A, HSP90AA1* mRNA levels were found during early embryo development. However, 8-cell embryos from vitrifried/warmed oocytes had significantly lower mRNA levels of *DNMT3A* and higher mRNA levels of *BAX* and *BCL2L1* compared to non-vitrified oocytes, regardless of the LIF treatment. *DNMT3A, KAT2A, HSPA1A* mRNA levels significantly increased in blastocysts produced from vitrified/warmed oocytes when compared to fresh control oocytes, while *BAX* and *BCL2L1* mRNA levels were significantly decreased, regardless of the LIF treatment. Changes of transcripts abundance in the oocytes and embryos following oocytes vitrification might be an early indicator of poor quality in good correlation with the developmental data to blastocyst stage.

2. INTRODUCTION

Oocyte cryopreservation has become a necessity in the last decades to preserve feminine genetic material of endangered species and of those of a great economic importance [1] . From the first attempts of vitrifiying bovine oocytes in 1992 [5], [6] , the vitrification protocol and the support devices used have changed considerably, trying to improve the three key factors in order to achieve a successful vitrification: high cooling/warming rate, high viscosity and small volume of the solution where the oocytes are suspended. However, fertilization rate and developmental competence of bovine cryopreserved oocytes still need to be improved. The peculiarities of the bovine oocyte, a large cell with low surface to volume ratio, high content of cytoplasmic lipid droplets [15] and low permeability coefficient partly due to a lack of aquaporin expression [17], make the oocyte more sensitive to chilling and freezing injury. Low outcomes in fertilization and blastocyst rate are associated to cooling and ice formation, what can result in premature release of cortical granules leading to zona hardening [21]–[23], disorganization and/or depolymerization of microtubules [10], [20], [226], abnormal mitochondrial appearance and distribution [22],[10], [26], chromosomal dispersal and DNA abnormalities [10], [27], [28] .

Cytokines and growth factors are naturally found in follicular fluid, promoting oocyte growth and survival, thus affecting the possible fertilization and embryo development. One of these cytokines is leukemia inhibitory factor (LIF), a 20kDa glycoprotein and a pleiotropic member from the interleukin (IL)-6 family of cytokines. The IL-6 family of cytokines consists of IL-6, IL-11, LIF, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC), IL-35 and IL-39; members that all share a common signal transduction receptor chain, the glycoprotein 130 receptor (GP130) (reviewed by [105]). LIF was first described to induce differentiation in M1 murine myeloid leukemic cells [167] and to maintain totipotency in embryonic stem cell lines [168] . LIF has been detected in human follicular fluid [136] and in bovine *LIF*-transcripts are naturally expressed from matured oocytes up to 16-cell stage [138] . The glycoprotein is present in granulosa cells of primordial follicles

where displays an essential function in follicular growth and development [137], [139], [140].

The addition of LIF into maturation media has been reported to promote oocyte maturation in many domestic species [141], [143], [144], [146], including the bovine among them [142] . It has been reported to improve developmental competence [141], [142], [149] and cleavage rate [145], [146] and confers better blastocyst quality in terms of total cell number [142], [149]–[151] , increasing inner cell mass count in some cases [146]. LIF ameliorates early embryo development [147], [148] and improves hatching rate [148], [150]. It has also been described as a fundamental factor for blastocyst implantation [152], [153], trophoectoderm proliferation [154] and invasion [155].

The interval between oocyte and embryonic gene transcription is ruled by the stored maternal factors in early mammalian development. Maternal effect genes (MEG) are responsible of this accumulation of transcripts and proteins that will drive through the maternal-to-embryonic transition (MET) (reviewed by [201], [202], [227]). Embryonic genome activation (EGA) occurs in different waves, and in bovine the major wave takes place at 8- to 16-cell stage [200]. Assessing gene expression at this time point could provide key information about changes in maturation or culture conditions.

As aforementioned, the members of IL-6 family of cytokines through GP130 receptor initiate a signal transduction cascade involving three different pathways: JAK/STAT, MAPK and PI3K (reviewed by [105], [159]). Genes involved in differentiation, survival, apoptosis and proliferation will be activated. Some of these target genes seem to be heat shock proteins (HSP) [221], [223], [224]. HSPs are molecular chaperones involved in cellular stress response [29] that are commonly used as cellular stress indicators [95], [228]. Mimicking the natural environment oocytes would find in physiological conditions through LIF supplementation, it could be a way to confer them resistance prior vitrification.

The present study aims to characterize the effect of LIF supplementation during bovine oocyte maturation on the vitrification process. Embryo development and gene expression will be assessed in order to elucidate the cytokine impact.

3. MATERIAL AND METHODS

3.1. Chemicals and suppliers

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated.

3.2. Oocyte collection and *in vitro* maturation

The *in vitro* protocols followed for maturation (IVM), fertilization (IVF) and culture (IVC) have been described previously by Rizos [163]. Ovaries from heifers (>12 months of age) and cows (>24 months of age) were transported from a local slaughterhouse in phosphate-buffered saline (PBS) at 35–37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2–10 mm follicles. Only COCs with three or more layers of cumulus cells and a homogeneous cytoplasm were selected for *in vitro* maturation. After three washes in modified Dulbecco's PBS (PBS supplemented with 0.036 mg/mL pyruvate, 0.05 mg/mL gentamicin and 0.5 mg/ml bovine serum albumin (BSA)), groups of up to 20 COCs were placed in 100 µL drops of maturation medium and cultured for 24 h at 38.5ºC in a 5% CO_2 humidified air atmosphere. The maturation medium consisted of TCM-199 supplemented with 10% (v/v) FBS, 10 ng/mL epidermal growth factor and 50 µg/mL gentamicin. LIF treated oocytes were matured with the same IVM medium additionally supplemented with human recombinant LIF (25 ng/mL). Immature oocytes in germinal vesicle stage (GV) and mature oocytes (MII) were collected for RNA extraction, reverse transcription and qPCR analysis.

3.3. Oocyte vitrification and warming

The vitrification/warming procedure was essentially described by Morató [10]. At 20h of IVM, COCs were randomly distributed in four groups: CF, fresh oocytes that worked as a maturation control; VIT, oocytes vitrified/warmed at 20h of IVM; and

LV, oocytes matured with 25 ng/mL LIF and vitrified/warmed at 20h of IVM. Vitrified groups were allowed to recover after warming so they reached 24h of IVM.

3.3.1. Vitrification protocol

The holding medium (HM) used to formulate the vitrification/warming solutions consisted of Hepes-TCM199 supplemented with 20% (v/v) FBS. Partially denuded oocytes were transferred into ES consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO) in HM for 9 min. Subsequently, oocytes were moved to the VS containing 15% (v/v) DMSO, 15% (v/v) EG and 0.5 M sucrose dissolved in HM. After incubating for 30–40 s, oocytes (up to 6) were loaded onto the Cryotop®, almost all the solution was removed to leave only a thin layer covering the oocytes, and then the Cryotop® was immediately plunged into liquid nitrogen. The entire process from exposure in VS to plunging was completed with less than 90 seconds.

3.3.2. Warming protocol

Vitrified oocytes were warmed by directly immersing the Cryotop® into WS1 (1M sucrose dissolved in HM) for 1 min. The oocytes were then transferred to WS2 (HM containing 0.5M sucrose) for 3 min. After a final wash in HM for 1 min, the oocytes were transferred back into maturation dishes and matured at 38.5ºC in humidified air with 5% CO_2 for approximately 2 h before being used for further experiments.

3.4. *In vitro* fertilization and embryo culture

In vitro matured oocytes were *in vitro* fertilized at 38.5ºC in a 5% CO_2 atmosphere. Briefly, frozen/thawed spermatozoa from Asturian bulls (ASEAVA, Llanera, Asturias, Spain) of proven fertility were used in all the experimental procedures. High motility and good morphology spermatozoa were obtained by centrifuging frozen/thawed sperm at 300 x g and room temperature for 10 min on a discontinuous gradient composed by 1 mL of 40% and 1 mL of 80% BoviPure (Nidacon Laboratories AB, Göteborg, Sweden) according to the manufacturer's specification. Viable spermatozoa collected from the bottom were washed with 3 mL of BoviWash (Nidacon International, Göteborg, Sweden) and pelleted by

centrifugation at 300 x g for 5 min. Spermatozoa were counted in a Neubauer chamber and diluted in an appropriate volume of fertilization medium (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 1 mg/mL heparin-sodium salt) to a final concentration of 1 x 10^6 spermatozoa/mL. 100 µL droplets of diluted sperm were made under mineral oil and 20 oocytes/droplet were co-incubated at 38.5ºC, 5% CO_2 and high humidity.

After 18–20 h, the presumptive zygotes were stripped of remaining cumulus cells by pipetting and cultured in groups of 20 in 20 µL drops of IVC medium, consisting of Synthetic Oviduct Fluid (Caisson Labs, Smithfield, USA) supplemented with 0.96 µg/mL BSA, 88.6 µg/mL Na-pyruvate, 2% non-essential amino acids, 1% essential amino acids, 0.5% gentamicin and 2% FBS under mineral oil. Presumptive zygotes were incubated at 38°C in a humidified 5% CO_2 and 5% O_2 atmosphere for 8 days. Embryo development was recorded on day 2 (cleavage), and days 7 and 8 (blastocysts) post-insemination (pi). Day 8 embryos were classified according to the degree of blastocoel expansion into three groups based on *Manual of the International Embryo Transfer Society* by Stringfellow & Seidel (1998): (1) non-expanded blastocysts: blastocoel volume less than one-half of the total volume of the blastocyst; (2) expanded blastocysts: blastocoel volume more than one-half of the total volume of the blastocyst; (3) hatching or hatched blastocysts: expanded blastocyst with an opened zona pellucida or lacking the zona pellucida. 2-cell (31-33 hpi), 8-cell (52-54 hpi) and day 8 blastocysts were harvested for RNA extraction, reverse transcription and qPCR analysis.

3.5. RNA extraction, reverse transcription and quantitative real-time PCR analysis

All the collected samples for gene expression analysis underwent the same RNA isolation protocol. Samples were washed three times with Dulbecco's PBS supplemented with 0.01% PVA at 38.5°C and then pipetted into 1.5 mL microtubes. Immediately, tubes were plunged into liquid nitrogen and stored at −80°C until further processed. GV and MII oocytes were pooled in groups of 20 and embryos in groups of 5. Poly-(A)-RNA was extracted using Dynabeads mRNA

Direct Extraction Kit (Invitrogen™, Oslo, Norway), following the manufacturer's instructions with minor modifications. For poly-(A)-RNA extraction, pooled samples were lysed in 50 µL of lysis buffer for 5 min with gently pipetting, and the fluid lysate was then hybridized with 10 µL pre-washed beads for 5 min with gently shaking. After hybridization, poly-(A)-RNA-bead complexes were washed twice in 50 µL of Washing Buffer A and two more times in 50 µL of Washing Buffer B. Next, the samples were eluted in 16 µL of Elution Buffer (Tris HCl) and heated to 70°C for 5 min. Immediately after extraction, 4 µL of qScript cDNAsupermix (Quanta Biosciences; Gaithersburg, MD, USA) were added and the reverse transcription (RT) reaction was carried out using oligo-dT primers, random primers, dNTPs and qScript reverse transcriptase. The RT reaction was performed for 5 min at 25°C, followed by 1 h at 42°C to allow the RT-PCR of mRNA and 10 min at 70°C to denature the reverse transcriptase enzyme. After RT, the cDNA was diluted with 25 µL of Tris HCl (elution solution).

Quantification of relative abundance of mRNA transcripts was performed by the qPCR method using a 7500 Real Time PCR System (Applied Biosystems, Foster City, California, USA). The qPCR reaction mix contained 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA), 1 µL of forward and 1 µL of reverse primers (Life Technologies, Madrid, Spain) and 2 µL of cDNA template. Nuclease-free water was added up to a final volume of 20 µL. The PCR amplification was carried out for one cycle of denaturation at 95°C for 10 min, 45 cycles of amplification with denaturation step at 95°C for 15 seconds, annealing step for 1 min at 60ºC (the appropriate annealing temperature of primers) and extension step at 72°C for 40 seconds. Fluorescence data were acquired during the 72°C extension steps. To verify the identity of the amplified PCR product, melting curve analysis and gel electrophoresis (in a 2% agarose gel containing 0.6 µg/mL ethidium bromide) were performed. The melting protocol consisted of heating the samples from 50 to 95°C, holding at each temperature for 5 seconds, while monitoring fluorescence. In each run there were three technical replicates from each of the three biological replicates per individual gene. Furthermore, negative controls for the template and for the reverse transcription were also included and amplified by PCR to ensure that no cross-contamination occurred.

Seven candidate genes (*DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*) for blastocysts and ten (*ZAR1*; *NPM2*; *DPPA3; DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*) for GV, MII, 2-cell and 8-cell embryos were used to perform quantitative PCR analysis in comparison with glyceraldehide-3-phosphate dehydrogenase (GAPDH) and H2A histone family member Z (H2AFZ) genes (endogenous control genes). The comparative threshold cycle (Ct) method was used to quantify relative gene expression levels and quantification was normalized to the endogenous control (housekeeping (HK) genes: GADPH and H2AFZ. Fluorescence data were acquired after each elongation step to determine the threshold cycle for each sample. The threshold cycle, which is set in the log-linear phase, reflects the PCR cycle number at which the fluorescence generated within a given reaction is just above background fluorescence. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative Ct method, the ΔCt value was determined by subtracting the mean between *GAPDH* and *H2AFZ* Ct values for each sample from the Ct value of each target gene of the sample for each replicate separately. Calculation of ΔΔCt involved the subtraction of the ΔCt value for the untreated (GV in experiment 1 and early control fresh embryos for experiment 2) from all the other ΔCt sample values. Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-(\Delta\Delta Ct)}$, the Livak and Schmittgen method. Primer sequences, amplicon size and GenBank accession numbers for each gene are provided in Table 1. The efficiency of primer amplification was 100%. Non-template controls were not amplified or returned a Ct value 10 points higher than the average Ct value for the genes. The experiment was repeated independently three times.

3.6. Statistical analysis

Statistical tests were performed using the statistical package IBM SPSS Version 25.0 for Windows (IBM Corp.; Armonk, NY, USA). Cleavage rates, blastocyst yields and relative transcript abundances were evaluated by a one-way analysis of the variance (ANOVA) followed by the post-hoc Sidak's test. Data are expressed as means ± standard error of the mean (SEM). Significance was set at $P \leq 0.05$.

3.7. Experimental design

EXPERIMENT 1. Characterize the effects of LIF added to the maturation media prior vitrification/warming on specific gene expression on oocytes and early embryos.

Upon oocyte collection, oocytes were randomly allocated in four groups: GV, CF, VIT and LV. GV oocytes were collected and immediately flash frozen for RT-qPCR analysis. Oocytes from CF, VIT and LV groups were *in vitro* matured. At 24h of IVM, groups of 20 MII oocytes from all groups were collected, and the rest were fertilized and cultured. Embryo development was allowed to continue until 31-33 hpi and 52-54 hpi, moment in which 2- and 8-cell embryos respectively were harvested.

EXPERIMENT 2. Characterize the effect of LIF added to the maturation media prior vitrification/warming on developmental competence and specific gene expression. After 24h of IVM, oocytes from all groups were *in vitro* fertilized and cultured. Cleavage rates were determined at 48 hpi and blastocyst rates on day 7 and day 8 pi (four replicates per group). At day 8, embryos were harvested for RNA extraction and RT-qPCR.

Symbol	Gene name	NCBI Reference Sequence	Primer set sequences (5'-3')	Length (nt)
BAX	BCL2 associated X, apoptosis regulator	NM_173894.1	F: GAGAGGTCTTTTTCCGAGTGGC R: TGTCCCAAAGTAGGAGAGGAG	237
BCL2L1	BCL2 like 1	BC147863.1	F: CCACTTAGGACCCACTTCTGAC R: GGGTGCTTCCTACAGCTACAGT	188
DNMT3A	DNA methyltransferase 3 alpha	NM_001206502.1	F: CCTCAGCTCCCCCTACTTATTC R: AGCTGTGAGCTTACTCCTGAGC	199
DPPA3	Developmental pluripotency associated 3	NM_001111108.2	F: TGGCTACTCTTCATCCCCTACA R: TCTAGGGTCCAGGTTGGGTT	230
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034.2	F: AGTCCACTGGGGTCTTCACTAC R: CAGTGGTCATAAGTCCCTCCAC	243
HDAC1	Histone deacetylase 1	NM_001037444.2	F: CTGAGGAGATGACCAAGTACC R: CCACCAGTAGACAGCTGACAGA	167
HSPA1A	Heat shock protein family A (Hsp70) member 1A	NM_203322.3	F: GCAGGTGTGTAACCCCATCA R: CAGGGCAAGACCAAAGTCCA	181
HSP90AA1	Heat shock protein 90 alpha family class A member 1	NM_001012670.2	F: GTGGAGACTTTCGCCTTCCA R: TGGTGAGGGTTCGATCTTGC	223
H2AFZ	H2A histone family, member Z	NM_174809.2	F: GCGTATTACCCCTCGTCACTTG R: GTCCACTGGAATCACCAACACTG	227
KAT2A	Lysine acetyltransferase 2A	XM_015468132.1	F: AGGATGTGGCTACCTACAAGG R: GCACCAGCTTGTCCTTCTCTAC	190
NPM2	Nucleophosmin / Nucleoplasmin 2	NM_001168706.1	F: GGACCTGTGTTCCTCTGTGG R: CTTCACTTGTTTGACGGGCG	153
ZAR1	Zygote arrest 1	NM_001076203.1	F: GGGAGATGCAAAGGCAAACG R: CCAAACAACAGCCTTCCACG	216

Table V-1. Primers used for reverse transcription–quantitative polymerase chain reaction

4. RESULTS

4.1. Experiment 1. Effect of LIF supplementation on gene expression of vitrified/warmed oocytes

The maternal effect genes analyzed were Zygote arrest 1 (*ZAR1*); nucleoplasmin 2 (*NPM2*) and developmental pluripotency associated 3 (*DPPA3*) (Figure 1). Quantification of *ZAR1* and *NPM2* did not bring up any significant difference between groups. Contrarily, *DPPA3* was significantly lower on matured oocytes that had been vitrified. Also, at 8-cell stage VIT group appeared to have a tendency to be significantly higher (P=0.077) in comparison to LV group.

RT-qPCR analysis of genes involved in epigenetic reprogramming is shown in Figure 2. Among the genes analyzed, just *DNMT3A* (DNA methyltransferase 3 alpha) exhibited significant differences at 8-cell stage embryos. Oocytes that had been vitrified/warmed at 20h of IVM significantly (P<0.05) decreased at 8-cell embryos in *DNMT3A*-transcript levels independently of LIF supplementation. *KAT2A* (lysine acetyltransferase 2A) and *HDAC1* (histone deacetylase 1) did not reveal any modification neither in mature oocytes nor early cleavage.

The results for the pro-apoptotic gene *BAX* (BCL2 associated X, apoptosis regulator) and the anti-apoptotic gene *BCL2L1* (BCL2 like 1) are shown in Figure 3. Control 8-cell stage embryos presented increased levels (P<0.05) of both *BAX* and *BCL2L1* gene products. This difference was not appreciated at the ratio calculation. Any other samples showed differences for any treatment.

There was no difference in gene products of the heat shock proteins analyzed (Figure 4). However, *HSPA1A* (heat shock protein 70 kDa) mRNA levels indicated an upward trend in mature oocytes, where the vitrified ones in absence of LIF, *HSPA1A* seemed to increase when compared to control (P=0.088) and to LV (P=0.067). No difference was found for *HSP90AA1* (Heat shock protein 90 alpha family class A member 1).

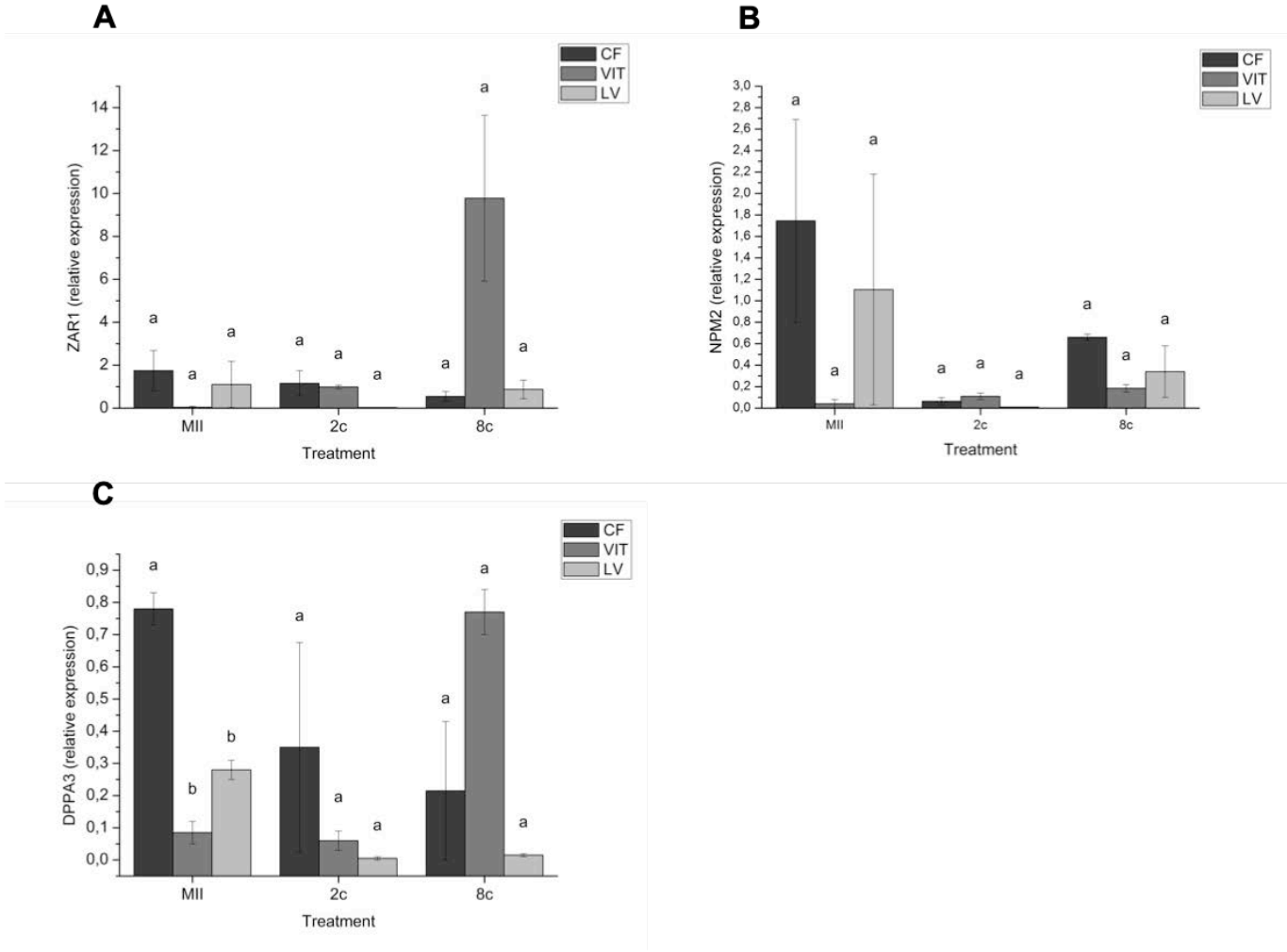


Figure V-1. Relative abundance (as mean ± SEM) of maternal effect genes. (A) *ZAR1*, (B) *NPM2* and (C) *DPPA3* in MII oocytes, 2- and 8-cell embryos from CF and vitrified groups. Different superscripts indicate significant differences (P<0.05).

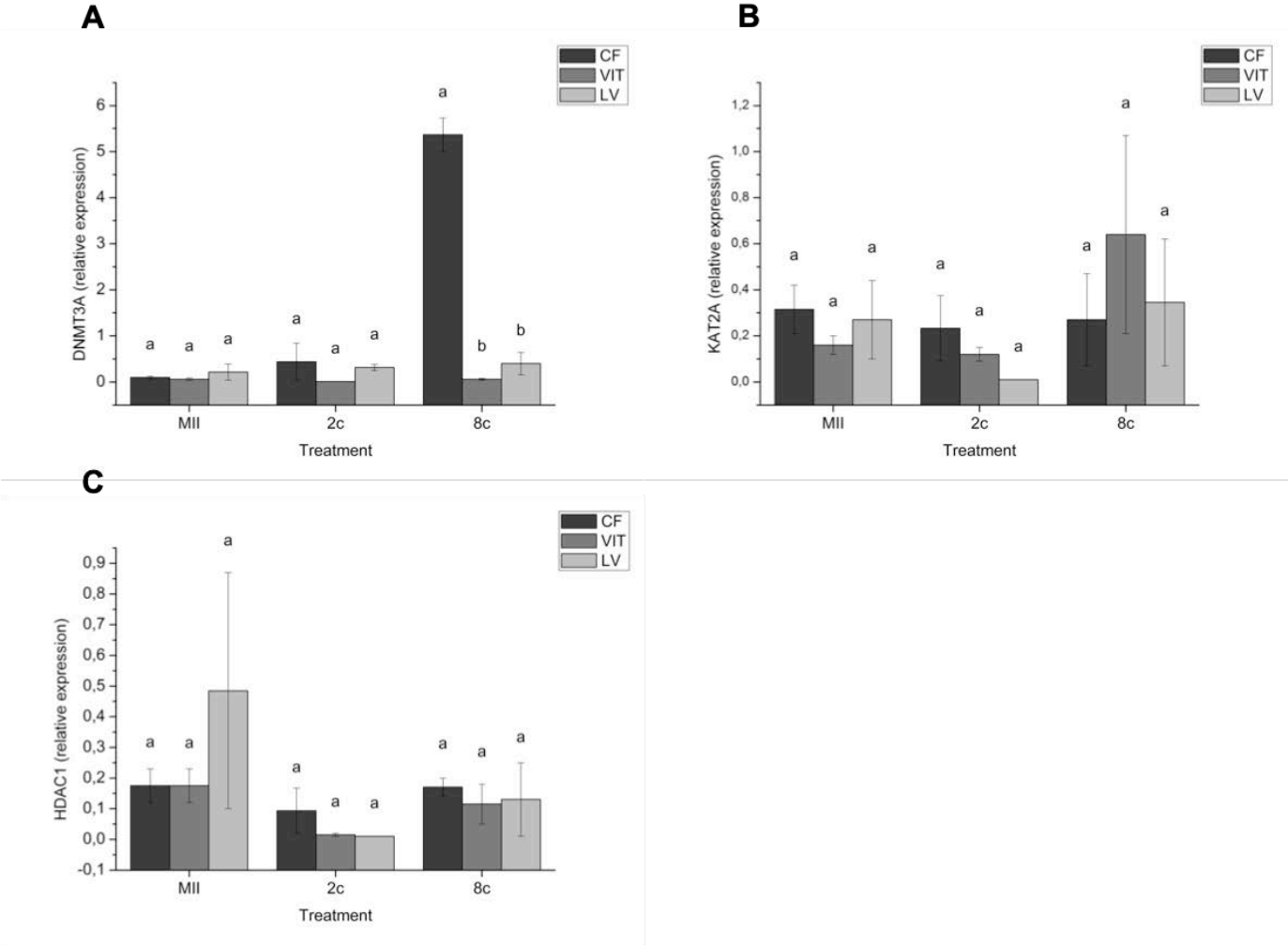


Figure V-2. Relative abundance (as mean ± SEM) of genes involved in epigenetic reprogramming. (A) *DNMT3A*, (B) *KAT2A* and (C) *HDAC1* in MII oocytes, 2- and 8-cell embryos from control and vitrified groups. Different superscripts indicate significant differences (P<0.05).

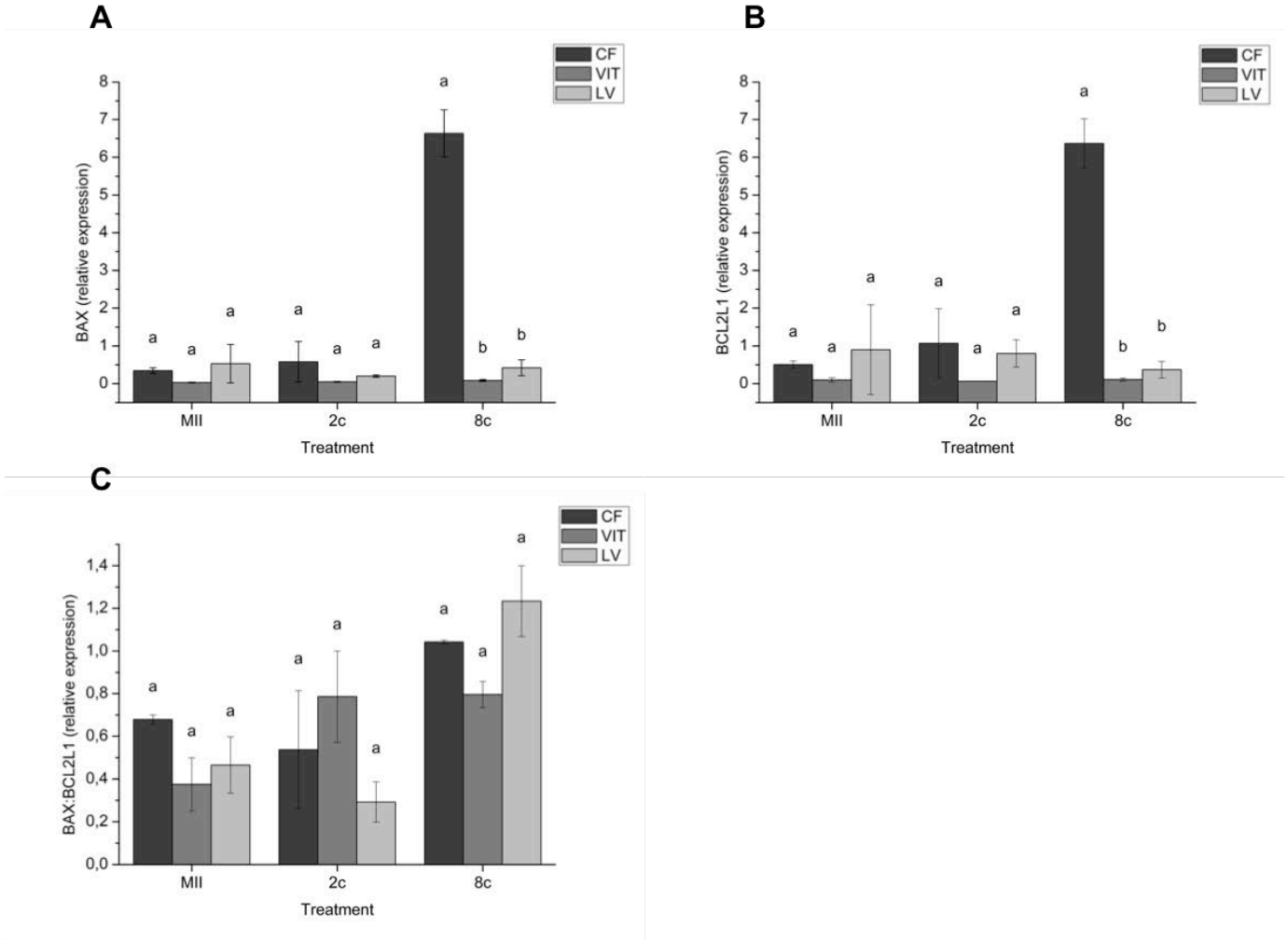


Figure V-3. Relative abundance (as mean ± SEM) of genes involved in apoptosis. (A) *BAX*, (B) *BCL2L1* and (C) *BAX:BCL2L1* ratio in MII oocytes, 2- and 8-cell embryos from control and vitrified groups. Different superscripts indicate significant differences (P<0.05).

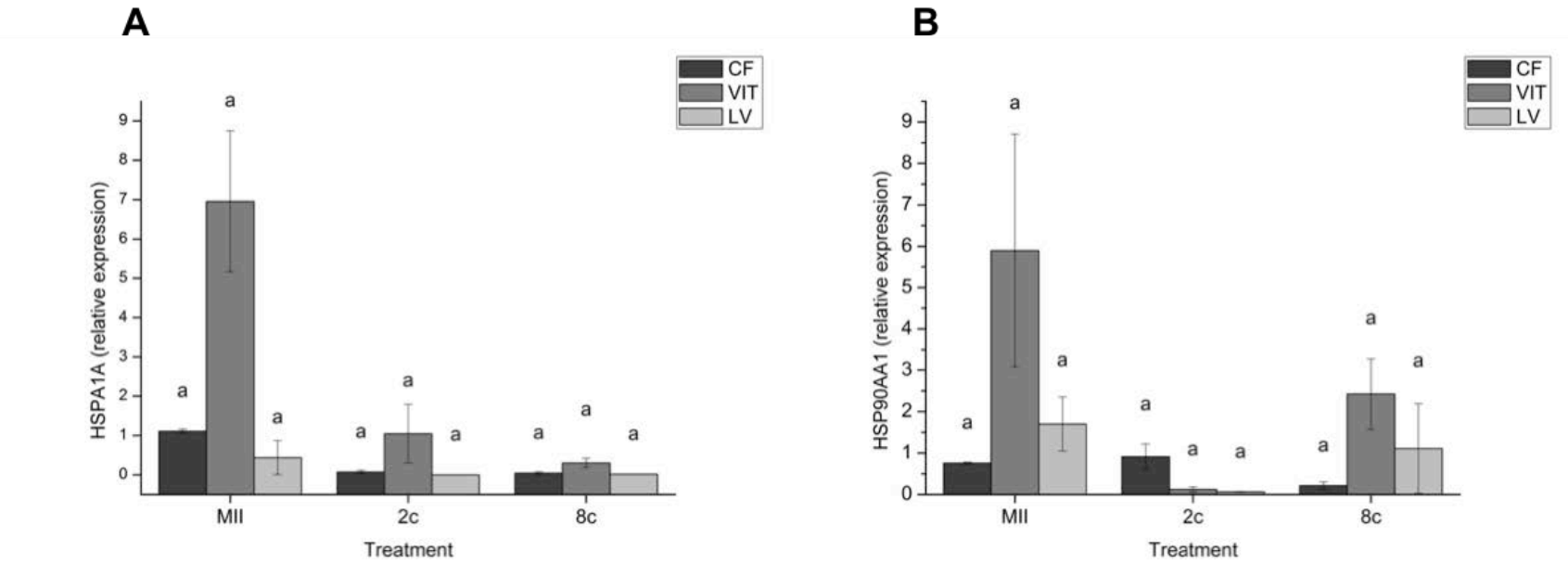


Figure V-4. Relative abundance (as mean ± SEM) of genes involved in heat stress. (A) *HSPA1A* and (B) *HSP90AA1* in MII oocytes, 2- and 8-cell embryos from control and vitrified groups. Different superscripts indicate significant differences (P<0.05).

4.2. Experiment 2. Effect of LIF supplementation on embryo development and gene expression of vitrified/warmed oocytes

The potential beneficial effects of LIF on the vitrification/warming process were evaluated on embryo development (Table 2). No, significant differences arouse neither in terms of cleavage rate nor blastocyst yield in day 7 and 8 of development. Nevertheless, on day 8 of development, embryos produced from vitrified oocytes exhibited a downward tendency compared to control (P=0.096). Meanwhile the embryos LIF-treated and vitrified showed no difference with respect to control and vitrified groups.

Concerning gene expression, the genes analyzed involved in epigenetics are shown in Figure 5. *DNMT3A* and *KAT2A* were increased at the blastocyst stage in those that had been previously vitrified. When embryos got to the expanded stage, mRNA levels for *DNMT3A* significantly decreased (P<0.05) in the both vitrified groups. In the case of *KAT2A* no more differences were observed in any other developmental stage. *HDAC1* did not show any alteration during embryo development under any treatment.

At blastocyst stage, embryos showed significantly increased (P<0.05) transcript levels for *BAX* and *BCL2L1* (Figure 6). In expanded embryos, those derived from vitrified oocytes significantly reduced *BAX* mRNA levels. Meanwhile for *BCL2L1*, lower levels were detected in expanded embryos produced from vitrified and LIF-vitrified oocytes when compared to control. No difference was observed for any of the apoptotic genes analyzed in hatched embryos. The *BAX*: *BCL2L1* ratio did not discover any imbalance in any developmental stage.

Heat shock protein 70 kDa (*HSPA1A*) mRNA quantification revealed a significantly increase (P<0.05) on those blastocysts produced from vitrified oocytes (Figure 7). During expansion, transcript levels were equalized between treatment groups, whereas once expanded, blastocysts from both vitrified groups presented lower amounts for HSPA1A. In the other hand, blastocysts derived from oocytes that underwent the vitrification process diminished in *HSP90AA1* expression levels, independently from LIF supplementation. In expanded

blastocysts, mRNA levels for *HSP90AA1* were significantly higher in the vitrified group when compared to the LIF-vitrified group. At the hatched stage, no difference was observed.

	n	Cleavage	D7 Blastocyst	D8 Blastocyst	D8 Blastocyst			
					n_{D8}	Non-expanded	Expanded	Hatched
Control	275	90.42 ± 1.82[a]	27.16± 4.23[a]	37.42 ± 5.42[a]	105	13.01 ± 3.47[a]	35.84 ± 5.44[a]	51.15 ± 7.60[a]
VIT	200	70.95 ± 8.28[a]	13.8 ± 6.97[a]	17.48 ± 5.08[a]	33	30.00 ± 15.28[a]	31.67 ± 4.41[a]	38.33 ± 10.93[a]
LV	223	72.81 ± 7.89[a]	16.70 ± 4.16[a]	21.85 ± 5.14[a]	47	16.05 ± 6.62	44.00 ± 8.75	39.96 ± 8.29

Table V-2. Development of oocytes supplemented with 25 ng/mL of LIF during oocyte maturation. n = total of fertilized oocytes. Different superscripts indicate significant differences (P<0.05).

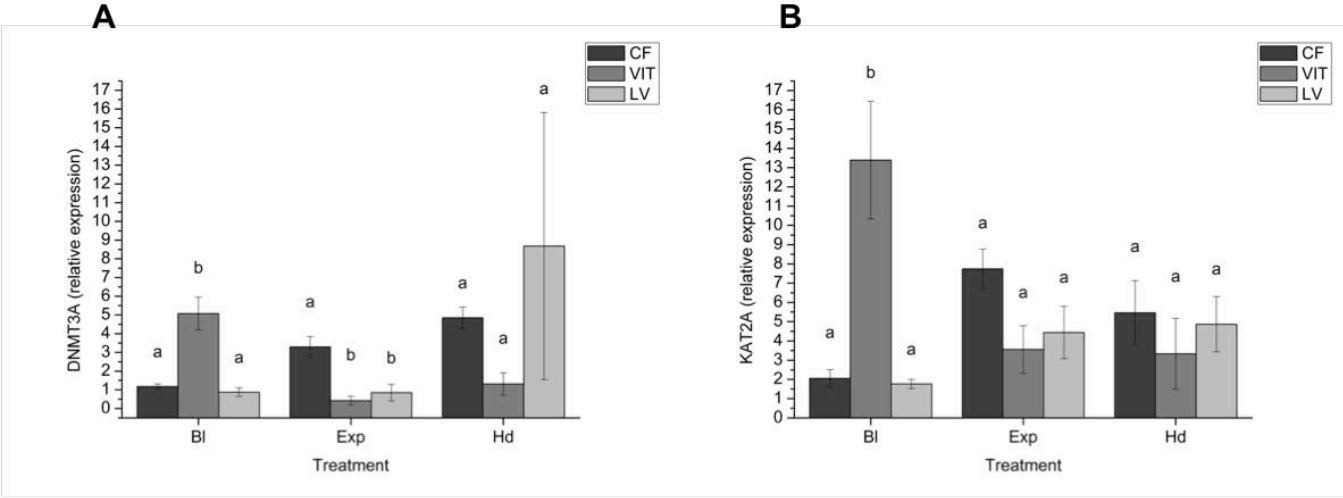


Figure V-5. Relative abundance (as mean ± SEM) of genes involved in epigenetic reprogramming. (A) *DNMT3A*, (B) *KAT2A* and (C) *HDAC1* in different blastocyst stages. Bl, blastocyst; Exp, expanded; Hd, hatched. Different superscripts indicate significant differences (P<0.05).

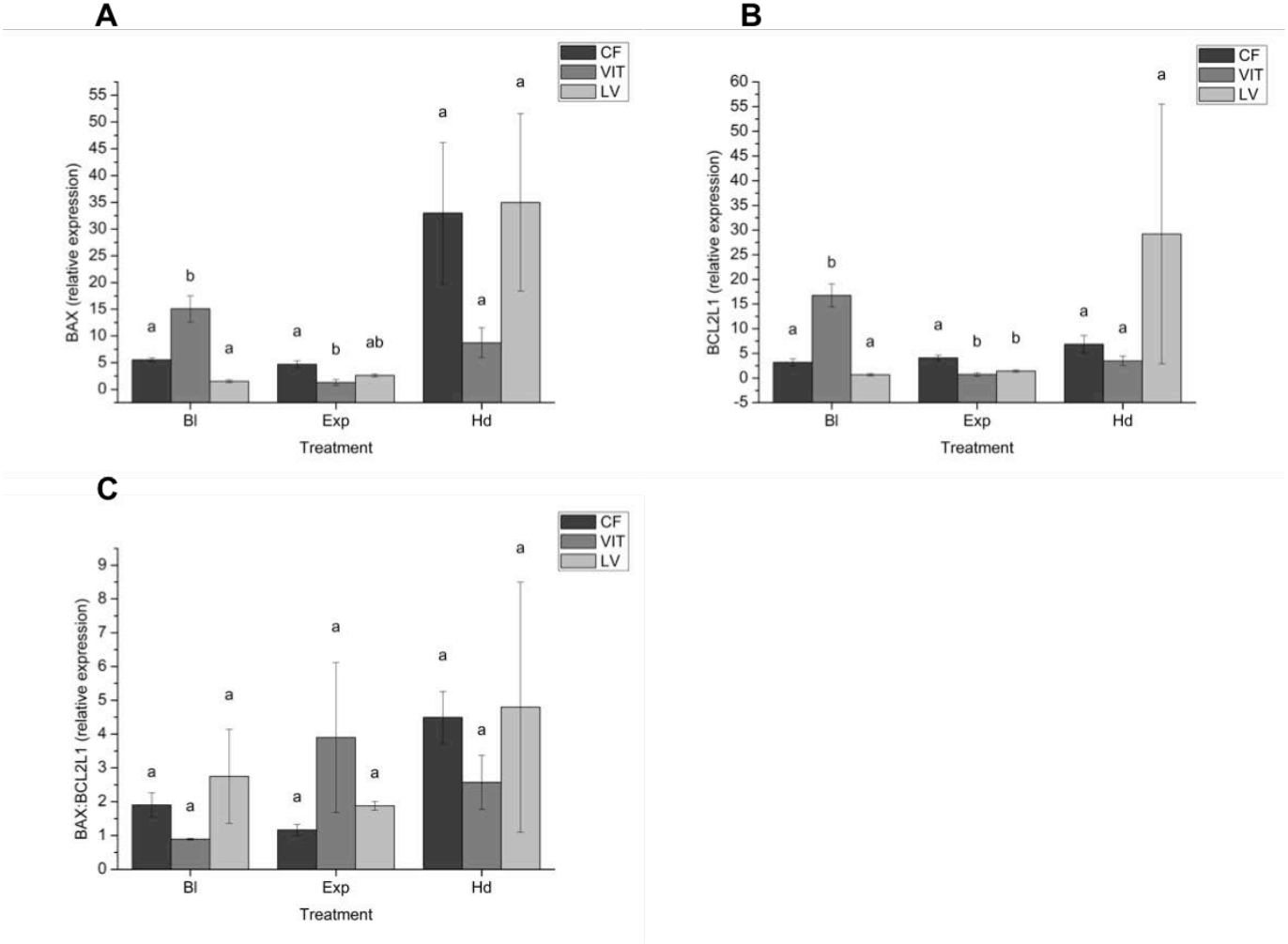


Figure V-6. Relative abundance (as mean ± SEM) of genes involved in apoptosis. (A) *BAX*, (B) *BCL2L1* and (C) *BAX:BCL2L1* ratio in different blastocyst stages. Bl, blastocyst; Exp, expanded; Hd, hatched. Different superscripts indicate significant differences (P<0.05).

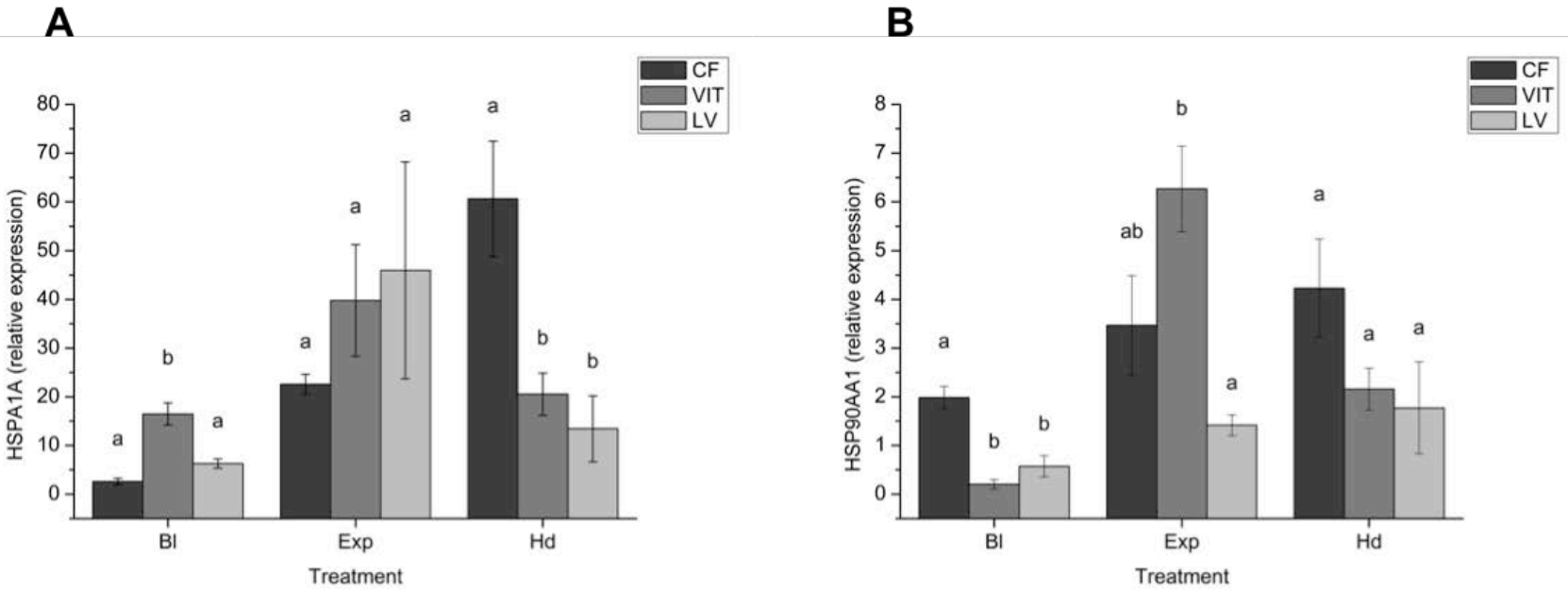


Figure V-7. Relative abundance (as mean ± SEM) of heat stress genes. (A) *HSPA1A* and (B) *HSP90AA1* in different blastocyst stages. Bl, blastocyst; Exp, expanded; Hd, hatched. Different superscripts indicate significant differences (P<0.05).

5. DISCUSSION

5.1. Experiment 1. Effect of LIF supplementation on gene expression of vitrified/warmed oocytes

During maternal-to-embryonic transition (MET), ZAR1 protein is involved in chromatin-mediated transcriptional regulation [229]. It has been demonstrated *ZAR1* is essential for MET, because embryos lacking *ZAR1* stop developing at 2-cell stage [230]. *NPM2* codifies for a histone chaperone that accumulates in the nucleus but not in the nucleolus and maintains heterochromatin architecture in oocytes and embryos [231], [232]. Regarding our data for *ZAR1* and *NPM2*, they did not present relevant variations between treatments. These results coincide with other studies in pig [205] and previous data we had obtained for oocytes matured in the presence of LIF without undergoing any cryopreservation procedure (data not shown). *DPPA3* has a function in methylation, as it keeps the methylation pattern on the maternal material [207]. *DPPA3* was downregulated on MII oocytes in both vitrified groups, apparently due to the harsh process of vitrification. Even though, when the impact of slow-freezing and vitrification on mRNA total content and specific gene expression was examined, cryopreservation techniques diminished the mRNA content, being vitrification less damaging. In this comparison, *DPPA3* never seemed to vary. 8-cell embryos from the VIT group, exhibited an upward tendency when compared to LV group. This decrease in *DPPA3* coincides with results in pig, where oocytes were treated with LIF in combination with other factors [206]. More studies are necessary in order to shed some light on the mechanistic of *DPPA3* and vitrification.

DNMT3A is a *de novo* methyltransferase that regulates gene expression by methylating CpG dinucleotides. Promoter gene regions are commonly rich in Cp[209], [210]. *DNMT3A* was found to be downregulated in both vitrified groups of 8-cell zygotes when compared to the control. Elevated levels of *DNMT3A* have been described as indicators of good quality [14], [211]. *KAT2A* (lysine acetyltransferase 2A) and *HDAC1* (Histone deacetylase 1) genes are also involved in epigenetics. Histone acetylation makes chromatin more accessible to the transcription

machinery [212] . Our data did not bring up any difference between treatments along embryo development neither for *KAT2A* nor *HDAC1*. A study done by McGraw and co-workers (2003) showed how mRNA levels for those genes did not change along normal bovine oocyte maturation and early embryo development; just *HDAC1* was upregulated in blastocysts. However, 2-cell embryos derived from vitrified oocytes increased in *KAT2A*-transcript levels [149].

BCL2 family members form a precisely organized signaling system that regulates apoptosis through permeation of the mitochondrial outer membrane. Gene *BAX* allows cytochrome c to release from mitochondria to the cytoplasm as well as the apoptosis inducing factor (AIF), driving the cell to apoptosis. Contrarily, *BCL2L1* works as an anti-apoptotic gene because it can bind to *BAX* at the outer membrane blocking the signaling cascade [233], [234]. In relation to our results, there was no detectable change but in control 8-cell stage embryos for *BAX* and *BCL2L1*. Because both of them increased, there ended up being no difference in terms of ratio, similar to the other stages analyzed. Alteration of the physiological process of maturation and/or development enhances the unfavorable *BAX* expression [101], [235] unbalancing *BAX: BCL2L1* ratio to the apoptosis pathway[236] .

Heat shock proteins (HSPs) are highly conserved cellular stress proteins that have been identified as a cell defense mechanism. Most of them are constitutively expressed at low levels but some can be upregulated in response to cellular stressors so to regulate protein folding, protect against protein aggregation and denaturation (reviewed by [30], [31]). Among the inducible ones, *HSPA1A* and *HSP90AA1* were those analyzed here. *HSPA1A* expression seemed to increase on vitrified MII oocytes when compared to control, a difference that LIF apparently mitigated. The other inducible HSP, *HSP90AA1*, did not show any variation. Vitrification did not prompt transcript levels of that in ovine oocytes. Similar results had been obtained in ovine vitrified oocytes [237]. In another study, vitrification with Cryotop® and solid-surface vitrification decreased *HSP90AA1* compared to control in MII oocytes and 2- to 8-cell zygotes.

5.2. Experiment 2. Effect of LIF supplementation on embryo development and gene expression of vitrified/warmed oocytes

DNMT3A was upregulated on vitrified-derived blastocysts. As development progresses, transcript levels diminish in both vitrified groups with the expansion of the blastocyst and, by the time the embryo hatches, differences among groups fade away. As stated above, elevated *DNMT3A* levels are associated of oocyte quality [14], [211]. So, it seems during early development, vitrification has a greater impact on the growing embryo, what can be surpassed as the development advances. There are no previous works, though, that have evaluated *DNMT3A* transcription levels at different time points during pre-implantational embryo development. In relation to histone modifications, no changes were found on *HDAC1*-transcript levels in embryos. *KAT2A* was upregulated on blastocysts produced from vitrified oocytes. This upregulation disappeared with development. There is some contradictory data around vitrification and its influence on acetylation levels, as in some cases seems to increase [14] and in others decrease [149] . Immunofluorescence studies on histone acetylation levels have been done, and they all agree on vitrification alters this epigenetic modification [238]–[240]. All this information exposes the necessity of more research around cryopreservation and histone acetylation influence on oocytes and embryo competency.

In terms of apoptosis, the same expression pattern for *BAX* and *BCL2L1* was characterized in embryos according to the stage of development. Neither in any of the genes mentioned, nor any treatment oocytes went through, caused a significant difference in reference to *BAX: BCL2L1* ratio. Which according to Duan et al (2005)[236] is responsible to lead the cell to apoptosis if *BAX* dominates, or to survival in case of *BCL2L1*. These results are in line with those obtained in embryo developmental rate, where no reduction in cleavage or blastocyst yield was evident. The observed declining tendency at day 8 vitrified embryos compared to control, might be associated to the expression patterns for *BAX* and *BCL2L1* observed at blastocyst and expanded stage.

mRNA level for *HSPA1A* at blastocyst stage was found to be increased, probably associated to the stress of vitrification. This was later attenuated until hatching. LIF can modulate *HSPA1A* expression through PI3K pathway. LIF activates AKT inducing HSF1 that ultimately will upregulate *HSPA1A* [220], [221]. LIF could have conferred oocytes a kind environmental adaptation prior vitrification by inducing *HSPA1A*, so cells did not need to generate a stress response and fight against the harmful vitrification process. In its turn, *HSP90AA1* decreased on vitrified groups at blastocyst stage by then increase during expansion just on those derived from VIT group. On other studies it has been shown how vitrification lowers HSP90 expression of other family members [241], [242]. More studies concerning *HSP90AA1* are necessary to elucidate the exact role on oocyte protection.

Chapter VI

General Discussion

Gamete cryopreservation, as many other reproductive technologies, has become a necessity for an efficient production of domestic animals, allowing the modification of animal breeding programs and offering a mean to maintain genetic diversity by banking important germplasm that could reinvigorate future populations[1]. Achieving a robust cryopreservation method would guarantee the preservation of feminine genetic material of endangered species and of those of a great economic importance. And that is the big challenge, freezing a huge peculiar cell without generating crystals and getting a thawed fully competent oocyte. Vitification is the most promising technique for oocyte cryopreservation. However, due to the peculiarities of the bovine oocyte, fertilization rate and developmental competence of bovine cryopreserved oocytes still need to be improved. During the vitrification process cells suffer cryodamage and ice formation, what leads to ultrastructural changes [10], [20]–[23], [26]–[28], [226] driving to maturation or development impairment.

For this purpose, in this thesis we have investigated different strategies to ameliorate the developmental competence of bovine oocytes after vitrification and warming processes. In general, we have observed that induced heat stress tolerance and the addition of cytokines prior vitrification have no effect on oocyte cryotolerance. Thus the aim of this general discussion is to integrate all the results reported in this thesis to highlight the most interesting findings.

Some studies have demonstrated that well-defined and properly applied stress, such as high hydrostatic pressure or osmotic, heat or oxidative stress, could increase tolerance of gametes and embryos to several *in vitro* and *in vivo* procedures [162]. When cells are subjected to various stress factors, they increase the production of a group of proteins called heat shock proteins (HSP). HSP are highly conserved chaperones expressed as a response to a raise in temperature and to other environmental factors to protect cells and they seem to be beneficial to the cells during cryopreservation, which is also a stress-inducing procedure [162]. For this reason, we designed an experiment where first, **a mild heat stress (HS) (1h at 41.5ºC) would be applied along IVM.** Different groups of oocytes represented every time point: **0, 4, 8, 12 and 16 h of IVM**. After 20 h of IVM,

oocytes from all groups were fixed in PFA to evaluate HSP70. In this way we could **determine the most suitable time point to apply HS treatment.** Second, based on the previous experiment, **oocytes would be subjected at 8h of IVM to a HS (1h at 41.5ºC) treatment prior vitrification.**

In the study presented in chapter II, a **heat stress treatment of 1h at 41.5ºC significantly (*P*<0.05) increased fluorescence intensity when applied at 8h of IVM**. Previous studies already observed a substantial increase of protein synthesis (approximately three-fold) in the time interval from 6 to 10 h of IVM [165], which occurs in parallel with the onset of germinal vesicle. When HS treatment was used to induce cryotolerance to oocytes prior vitrification, no positive effect was observed in terms of cleavage and blastocyst rate. However, HS treatment did not result being detrimental for oocyte competence in terms of blastocyst yield. This is in concordance with the study from [80] who after applying a similar HS procedure at 20-22h of IVM did not find deleterious effects on development to the blastocyst stage.

MicroRNAs have been shown to be important regulators of gene expression during oocyte maturation and embryo development [160], [178], [179]. The roles of miRNAs in the growth and maturation of oocytes and their surrounding follicular cells have not been widely characterized. However, through *in silico* and *in vitro* examination of specific target genes, it appears likely that these molecules participate widely in the regulation of apoptosis, proliferation and signaling pathways that are important for follicular growth and oocyte maturation [180]. In particular, the miR-21 has been observed to change profoundly during oocyte maturation and early embryogenesis [179], [181], [182]. A limited number of studies have suggested potential roles for LIF in the specific regulation of miR-21 expression [160], but a more comprehensive examination of the roles of the IL-6 family and the involvement in the JAK/STAT signal transduction pathway in the control of miRNA expression in oocytes and cumulus cells has not been undertaken.

In order to identify potential roles that miRNAs may play in follicular biology in response to cytokine signaling, the present study aimed to **determine whether the IL-6 family members LIF (25 gn/mL), IL-6 (10 ng/mL) or IL-11 (5 ng/mL) induced changes in the expression of miR-21 and other key miRNAs in bovine cumulus cells and oocyte cultured *in vitro* as cumulus oocyte complexes.** LIF showed higher expression of miR-21 in both cumulus cells and oocytes, an expected result observed in previous studies [160]. Some studies have proposed that **miR-21 should be considered a potential biomarker for oocyte quality, as it has been reported that increased miR-21 expression is associated with a reduction in apoptosis** [216]. Surprisingly, despite the fact that IL-6 activates STAT3 phosphorylation in myeloma cells [185], we did not observe any significant increase in miR-21 with other cytokines examined in either cumulus cells or oocytes. miR-155 expression was found to be increased in cumulus cells control maturation conditions, while no noticeable change was found in cumulus cells from COCs treated with LIF, IL-6 or IL-11. **A consistent, but non-significant trend towards increased levels of miR-155 was observed in oocytes from LIF and control groups in addition to those undergoing normal in vitro maturation.** Functionally, miR-155 is one of the most prominent miRNAs in inflammatory processes, and it is associated with ROS induction through the suppression of antioxidant genes [193]. After induction through TLR4 miR-155 [194] act as negative feedback regulators of inflammation, inhibiting TAB2 [197] a kinase in the NF-κB pathway. Therefore, miR-155 exerts anti-inflammatory properties by down-regulating IL-6 and other target genes. In the present context our data suggest that levels of miR-155 tend to be lower when IL-6 and IL-11 are added to the maturation medium. Given the overlap between inflammation and follicular events, the anti-inflammatory role of miR-155 could thereby provide one potential explanation for the elevated levels in untreated cumulus cells, and in control and LIF-treated oocytes where such feedback has not occurred. In this same study, **IL-11 significantly increased miR-146a levels in oocytes.** Our results of miR-146a on oocytes may be due to activation of pathways other than STAT3, as characterized by Li et al. (2012)[186]. They first characterized the **IL-11 signaling pathway that leads to an activation of NF-κB, a transcription factor** associated with IL-6 expression [243]. IL-11 activates via JAK the PI3K/AKT

pathway. AKT phosphorylates IKK, which in turn targets IκB for ubiquitination, releasing **NF-κB,** which then **translocates into the nucleus and interacts with the promoter region inducing miR-146a expression**. This activation of an alternative signaling pathway may help explain the selective effect of IL-11 on miR-146a in the present context. **LIF, IL-6 or IL-11 did not affect the expression of miR-34c a key member of the larger family members** (34a,b,c) important for gametogenesis and embryo development [186]–[189]. miR-34c has been detected in bovine germinal vesicle, mature oocytes and 2-cell embryos [190]. It has been described **miR-34c expression is induced by p53 tumor suppressor, leading to an inhibition of *BCL2* expression, driving cells to apoptosis** [192]. **It is therefore perhaps not surprising LIF, IL-6 or IL-11 did not induce the expression of miR-34c, as this would likely be more reflective of cellular stress.**

Considering the beneficial effects attributed to LIF in terms of oocyte maturation and embryo development [142], and the results obtained in chapter III in relation to miR-21 that help confirming LIF as a treatment to enhance oocyte quality. In chapter IV, **LIF 25 ng/mL supplementation during maturation was assessed on embryo development and gene expression in oocytes and pre-implantational embryos**. The microenvironment provided to oocytes will affect both oocyte and embryo gene expression [100], [101]. **In a first experiment, germinal vesicle oocytes, MII oocytes, 2- and 8-cell embryos were preserved for the analysis of mRNA levels** of ten candidate genes (*ZAR1*; *NPM2*; *DPPA3*; *DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*). **No changes in expression of *ZAR1*, *NPM2*, *DPPA3*, *DNMT3A*, *KAT2A*, *HDAC1*, *BAX*, *BCL2L1* and *BAX*:*BCL2L1* ratio were observed for MII, 2- and 8-cell stage embryos.** Our results concerning maternal effect genes (*ZAR1*, *NPM2* and *DPPA3*) did not present any significant differences between treatment groups. These data is supported by previous results in pig [205] were LIF supplementation during maturation did not induce any change in MII oocytes, 2- and 8-cell embryos. However, **maturation with LIF significantly ($P<0.05$) increased the expression of *HSPA1A* in 2-cell stage embryos or the expression of *HSP90AA1* in 8-cell stage embryos**. The induction of both HSPs seem to be orchestrated through PI3K/AKT pathway,

which is activated by LIF [220], [221]. Embryonic genome activation (EGA) takes place in two differentiated waves in bovine, the major one around 8-cell stage [201], [203], moment at which we could appreciate changes in expression for the stress-inducible heat-shock proteins. Such a delicate moment for embryos allowed to observed a tendency for increasing transcript levels on 8-cell treated embryos for *DNMT3A* and *HDAC1*, genes involved in epigenetics reprogramming. Elevated levels for *DNMT3A* have been related to cryotolerance [211].

In a second experiment, cleavage rates and blastocyst rates were assessed and day 8 blastocysts were harvested for the analysis of mRNA levels of seven candidate genes (*DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*). No effects of LIF supplementation during maturation were observed when cleavage rate or blastocyst yield were assessed. When gene expression was analyzed on day 8 blastocysts, no changes in mRNA expression were observed for *KAT2A*, *HDAC1*, *HSP90AA1*, *BAX*, *BCL2L1* and *BAX*:*BCL2L1* ratio. **However, expanded and hatched embryos from oocytes *in vitro* matured with LIF showed a significant decrease ($P<0.05$) for *DNMT3A*-transcript levels while HSPA1A-transcript levels.** Frezzato *et al.* (2019)[221] described two different models of how HSF1 is regulated by HSP70 protein. Elevated levels of HSP70 increased levels of active AKT that inhibits GSK3A/B by phosphorylation. This protein works as an inhibitor for HSF1, that now its inhibition leads to an upregulation of *HSPA1A*. When HSP70 is present in low levels, there is an upregulation of MAPK signaling pathway. Activated ERK can phosphorylate HSF1 causing inhibition and, therefore, preventing *HSPA1A* transcription.

In chapter V, we hypothesized that *in vitro* maturation conditions and vitrification have an effect on gene expression at various embryos developmental stages, which may affect their developmental potential. Here, we examined the developmental capacity of vitrified/warmed bovine oocytes in vitro matured in a maturation medium supplemented with leukemia inhibitory factor (LIF). First, **we evaluated changes in mRNA profiles for ten genes (*ZAR1*; *NPM2*; *DPPA3; DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*) in GV, MII, 2-cell and 8-cell embryos.** No differences in the *ZAR1, NPM2, KAT2A, HDAC1, HSPA1A, HSP90AA1* mRNA levels were found during early embryo development. However,

8-cell embryos from vitrifried/warmed oocytes had significantly lower mRNA levels of *DNMT3A* compared to non-vitrified oocytes, regardless of the LIF treatment. Elevated levels of *DNMT3A* have been described as indicators of good quality [14], [211] (Lopera-Vasquez *et al.*, 2015; Arcaron et al., 2019).

Second, seven genes (*DNMT3A*; *KAT2A*; *HDAC1*; *BAX*; *BCL2L1*; *HSPA1A*; *HSP90AA1*) in day 8 blastocysts. No significant differences arouse neither in terms of cleavage rate nor blastocyst yield. *DNMT3A, KAT2A, HSPA1A* mRNA levels significantly increased in blastocysts produced from vitrified/warmed oocytes when compared to fresh control oocytes, while *BAX* and *BCL2L1* mRNA levels were significantly decreased, regardless of the LIF treatment. There is some contradictory data around vitrification and its influence on acetylation levels, as in some cases seems to increase [14] and in others decrease [149]. Immunofluorescence studies on histone acetylation levels have been done, and they all agree on vitrification alters this epigenetic modification [238]–[240]. All this information exposes the necessity of more research around cryopreservation and histone acetylation influence on oocytes and embryo competency. The changes in expression patterns for *BAX* and *BCL2L1* did not affect ultimately *BAX: BCL2L1* ratio. Which according to Duan et al (2005)[236] is responsible to lead the cell to apoptosis in case *BAX* unbalances the ratio. Changes in expression for *HSPA1A* are probably due to LIF effect through PI3K pathway as stated above. LIF could have conferred oocytes a kind environmental adaptation prior vitrification by inducing *HSPA1A*, so cells did not need to generate a stress response and fight against the harmful vitrification process. In its turn, *HSP90AA1* decreased on vitrified groups at blastocyst stage by then increase during expansion just on those derived from VIT group. On other studies it has been shown how vitrification lowers HSP90 expression of other family members [241], [242]. More studies concerning *HSP90AA1* are necessary to elucidate the exact role on oocyte protection.

Chapter VII

Conclusions

1. A mild heat stress treatment of 1h at 41.5ºC is not over the limit of tolerance of bovine oocytes and it is able to stimulate HSP70.

2. A heat stress applied at 8h of IVM does not negatively affect in vitro embryo development up to blastocyst stage. However, any beneficial effect on embryo developmental competence could be observed when applied prior to vitrification.

3. LIF, as opposed to other IL-6 family members, induces miRNA profiles most widely associated with oocyte quality in cattle. It has the potential to be used as a treatment to enhance oocyte competency.

4. The inclusion of LIF ng/mL in the bovine *in vitro* maturation system influences mRNA expression of two stress-inducible heat-shock proteins (*HSPA1A* and *HSP90AA1*) in 4 or 8-cell embryos, coinciding with the embryonic genome activation. Also, maturing oocytes under LIF causes a decrease in mRNA expression of *DNMT3A* and *HSPA1A* in bovine blastocysts *in vitro.*

5. LIF appears to be a good inductor of cryotolerance. Oocytes matured with 25 ng/mL that were later vitrified developed normally until hatching stage with good hatching capacity. Through PI3K pathway LIF can induce *HSPA1A*, what presumably helps counteracting apoptosis.

Chapter VIII

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