



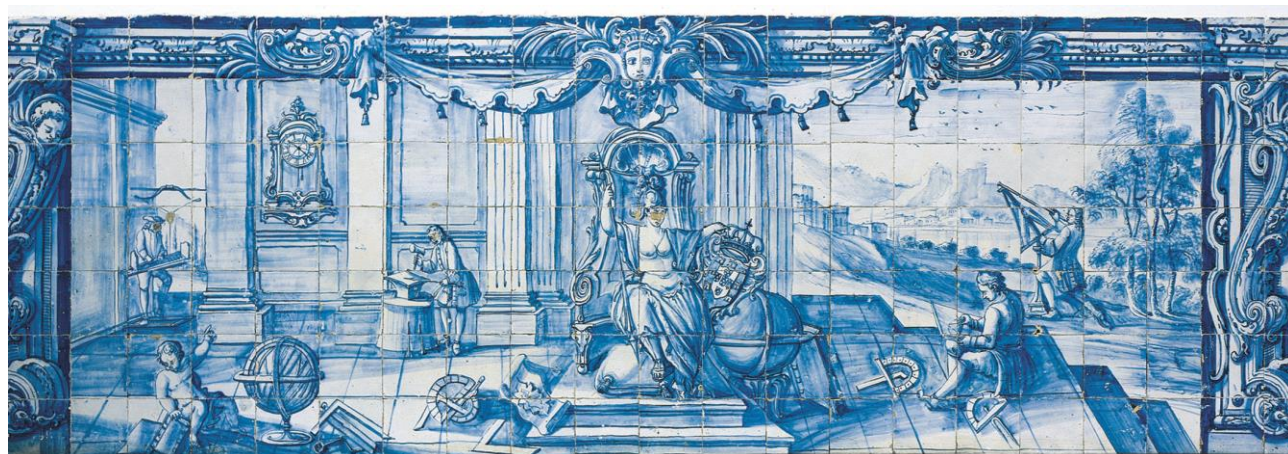
Cryopreservation of sheep embryos produced *in vitro*: the effect of protocols of lipid reduction

Ricardo Jorge da Costa Trindade Palmeiro Romão

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Ciências Veterinárias

ORIENTADORES: *Prof^a Doutora Rosa Maria Lino Neto Pereira*
Prof. Doutor Carlos Eugénio Plancha dos Santos

ÉVORA, AGOSTO 2014



Cryopreservation of sheep embryos produced *in vitro*: the effect of protocols of lipid reduction

Ricardo Jorge da Costa Trindade Palmeiro Romão

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Ciências Veterinárias

ORIENTADORES: *Prof^a Doutora Rosa Maria Lino Neto Pereira*
Prof. Doutor Carlos Eugénio Plancha dos Santos

ÉVORA, AGOSTO 2014



INDEX

Abstract	iii
Resumo	iv
Dedication	v
Funding acknowledgments	vi
Personal acknowledgments	vii
Figure index.....	ix
Table index.....	xi
List of abbreviations	xii
1 Preamble	1
2 Introduction.....	4
2.1 Application	5
2.2 <i>In vitro</i> embryo production.....	7
2.2.1 Collection of oocytes	8
2.2.2 <i>In vitro</i> maturation (IVM).....	9
2.2.3 <i>In vitro</i> fertilization.....	10
2.2.4 <i>In vitro</i> culture	10
2.3 Cryopreservation of sheep embryos	11
2.3.1 Slow freezing	12
2.3.2 Vitrification	13
2.3.3 Vitrification in OPS.....	14
2.3.4 Cryoprotectants	15
2.3.5 Warming of thawed embryos.....	16
2.4 Methods for improving cryopreservation of sheep embryos	16
2.5 Evaluation of cryopreserved embryos	19
3 Published preliminary results	22
3.1 <i>In vitro</i> production of ovine embryos using fresh semen can improve blastocyst quality in Portuguese Merino breed	23
3.2 Cryopreservation of ovine <i>in vitro</i> produced embryos using centrifugation and cytochalasin D.....	24
4 Published papers.....	26
4.1 Evaluation of two methods of <i>in vitro</i> production of ovine embryos using fresh or cryopreserved semen.....	27
4.1.1 Introduction	29
4.1.2 Materials and methods	30
4.1.3 Results.....	34
4.1.4 Discussion	36
4.1.5 Conclusion.....	38
4.1.6 References	38
5 Papers submitted to publication	41
5.1 Ultrastructural characterization of fresh and vitrified <i>in vitro</i> and <i>in vivo</i> produced sheep embryos.....	42
5.1.1 Introduction	44
5.1.2 Materials and methods	44
5.1.3 Results.....	47
5.1.4 Discussion	48
5.1.5 References	50

5.2	Cryopreservation of <i>in vitro</i> produced sheep embryos: effect of different protocols of lipid reduction	57
5.2.1	Introduction	59
5.2.2	Materials and methods	60
5.2.3	Results	63
5.2.4	Discussion	66
5.2.5	References	70
5.3	Ultrastructure of <i>in vitro</i> produced sheep embryos: effect of <i>trans</i> -10 <i>cis</i> -12 conjugated linoleic acid (CLA) in fresh and cryopreserved blastocysts.....	74
5.3.1	Introduction	76
5.3.2	Materials and methods	77
5.3.3	Results	80
5.3.4	Discussion	81
5.3.5	References	84
6	Discussion and conclusions	91
7	References	96

Abstract

In vitro production of sheep embryos and cryopreservation are developing reproductive technologies that haven't reached yet the expected results for routine application, mainly by the reduced cryotolerance when compared to *in vivo* derived ones. A series of experiments were developed searching for alternative methods of production and cryopreservation of this source of embryos. It was concluded that *in vitro* fertilization with fresh ram semen improves quality of *in vitro* produced sheep embryos when conjugated with a medium containing pyruvate and FSH. Also different protocols of lipid reduction can be successfully applied, when using a combination of cytochalasin D and centrifugation or by adding *trans*-10 *cis*-12 conjugated linoleic acid isomer (CLA) in culture, as they increased cryotolerance of embryos and also the production rates. In addition it was described the ultrastructural characteristics of *in vitro* produced sheep embryos and those cultured in the presence of CLA, either fresh or cryopreserved.

Key words: sheep; embryo; *in vitro*; cryopreservation; delipidation

Criopreservação de embriões de ovino obtidos *in vitro*: o efeito de protocolos de redução lipídica

Resumo

A produção *in vitro* de embriões ovinos e respectiva criopreservação são biotecnologias reprodutivas em desenvolvimento que ainda não atingiram os resultados esperados para serem aplicados rotineiramente, sobretudo pela menor criotolerância destes embriões quando comparados com os produzidos *in vivo*. Realizaram-se uma série de experiências procurando métodos alternativos de produção e criopreservação deste tipo de embriões. Concluiu-se que a fertilização *in vitro* com sêmen fresco de carneiro aumenta a qualidade dos embriões produzidos quando conjugada com meio contendo piruvato e FSH. Também se concluiu que é possível aplicar com sucesso protocolos de redução lipídica usando uma combinação de citocalasina D com centrifugação ou adicionando o isômero conjugado *trans*-10 *cis*-12 do ácido linoleico (CLA) ao meio de cultura, responsáveis pelo aumento da criotolerância dos embriões e maiores taxas de produção. Adicionalmente descreveram-se as características ultraestruturais dos embriões ovinos produzidos *in vitro* e dos produzidos em presença de CLA, quer frescos quer congelados.

Palavras-chave: ovino; embrião; *in vitro*; criopreservação; delipidação

Dedication

To Afonso, André and Ana Isabel,
who came to light together with this thesis,
wishing they be the carriers of our ancestors'
best virtues through the future.

Funding acknowledgments

This work was supported by Funds from the Portuguese Foundation for Science and Technology (PPTDC/CVT/98607/2008) and PhD Grant (SFRH/BD/37853/2007).

This work is funded by FEDER Funds through the Operational Programme for Competitiveness Factors - COMPETE and National Funds through FCT - Foundation for Science and Technology under the Strategic Project PEst-C/AGR/UI0115/2011.

The work of Mário Sousa and Elsa Oliveira were partially supported by UMIB-National Funds through FCT-Foundation for Science and Technology, under the Pest-OE/SAU/UI0215/2014.

Participation in the Conference ESDAR 2013 was supported by ICAAM – “Instituto de Ciências Agrárias e Ambientais Mediterrânicas”- Universidade de Évora – Núcleo da Mitra, Ap. 94, 7002-554, and funded by FEDER Funds through the Operational Programme for Competitiveness Factors - COMPETE and National Funds through FCT - Foundation for Science and Technology under the Strategic Project PEst-C/AGR/UI0115/2011.

Personal acknowledgments

To Dr. Rosa Lino Neto, by accepting be my PhD advisor, for all the time spent with this work that provided me the necessary knowledge and other tools that I shall use in the future. I appreciated especially the patience, goodwill and accuracy applied during the last 6 years. Maybe because I know that attending my expectations, hesitations, and difficulties was not an easy task. Now I know we succeeded.

To Dr. Carla Marques, Dr. Maria Conceição Baptista, Dr. João Pedro Barbas, Dr. António Horta and all other colleagues and staff of Estação Zootécnica Nacional (EZN), who allowed the production of all this work, wishing them all continue to struggle for new research in Animal Science that is needed for Portugal and other countries. All this colleagues and friends welcomed me since the first day and are responsible that I see EZN also as one of my references through the future.

A special thanks to Dr. Nuno Carolino for putting the pieces of our work together whenever needed with his statistics that was essential in all this research. Also for the optimistic however realistic and professional way of acting that must be an example for all Animal Science students as me.

To Dr. Carlos Plancha, my co-adviser, and Patricia Rodrigues that opened me the possibility of sharing their experiences and knowledge in Reproductive Biology, and also allowed the use of the facilities of the Faculty of Medicine of University of Lisbon.

To Dr. Mário Sousa and lab technician Mrs. Elsa Oliveira for all the hard work in processing and preparing the material for electron microscopic evaluation of embryos. It was also patent the competence of all the team of the Department of Microscopy, Laboratory of Cell Biology, Multidisciplinary Unit for Biomedical Research-UMIB of the Institute of Biomedical Sciences Abel Salazar and also the possibility of cooperation with other research teams. This is very important in the multidisciplinary approach of making science, as I believe.

To Dr. Carlos Bettencourt and all the staff of Centro de Experimentação do Baixo Alentejo, for the possibility of using sheep flock and facilities that, once again, were put into service for research purposes.

To all the staff and colleagues of the slaughterhouse where oocytes have been collected, MATREZE, Matadouro Regional do Zêzere, in Pedrogão Grande. In addition to the possibility of refreshing the view of meat inspection activity, it was a pleasure to review some of you again after the times of Veterinary Faculty.

To all my colleagues in Universidade de Évora that shared many of my difficulties, and cooperated also in my absence. My wish is to return with new strengths and skills to contribute, all together, for the future of our University.

To all my students, some of them already graduated during this long working period, knowing that much of this effort is also for them. Shall I have the opportunity and ability to contribute more and more for the expected challenges in education and research with students.

To all my other friends not mentioned, knowing that all friends are important, for being present in my life and because I know they are there whenever needed.

To Elisa Bettencourt, who is present since ever. In addition to our friendship (either in good and bad moments) that can positively influence decisions, she is a special inspiration as an example of dedication to work and duty, grounded in values as simplicity, honesty and determination.

To my family, headed by my parents, Guida and Joaquim, who provided me the values of life, the gift of education and the tools for building the same for their grandchildren.

To my wife, Alexandra, whose female side complements my existence and to whom I owe some of the time spent in this stage of my life. I know this was not a spent time but, instead, a tonic for new steps in our lives, with other challenges and hills to climb next.

Figure index

- Figure 1:** sample of poster (100x70 cm) presented in the ESDAR conference 2013, 12-14 September Bologna, Italy..... 25
- Figure 2:** Percentual distribution of embryo (n=279) morphological quality [grade 1 embryos (G1); grade 2 embryos (G2); grade 3 embryos (G3)] in the four groups according to maturation method (M1: maturation medium containing EGF and M2: medium containing EGF, pyruvate and FSH) and type of semen used: fresh (F) or frozen–thawed (FT) ($a \neq b$, $P=0.007$). 35
- Figure 3:** Ultrastructure of fresh *in vitro* ovine embryos at early blastocyst stage. Outer (A,C,E) and inner cells (B,D,F), cells. Microvilli (Mv), perivitelline space (PvS), cell debris (*), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), lipid droplets (L), fusion (white open arrow) of light (LV) and dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc). 53
- Figure 4:** Ultrastructure of fresh *in vivo* ovine embryos at early blastocyst stage. Outer (A,C) and inner cells (B,D), cells. Microvilli (Mv),perivitelline space (PvS); fusion of light vesicles (white arrow), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), rough endoplasmic reticulum (black arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), lipid droplets (L),secondary lysosomes (Ly). 54
- Figure 5:** Ultrastructure of OPS vitrified *in vitro* sheep embryos at early blastocyst stage fixed 1h after thawing. Blastocysts were classified as grade 2 (A and B) and grade 3 (C and D) in semithin sections. Zona pellucida (ZP) perivitelline space (PvS), microvilli (Mv), blastocoelic cavity (b), nuclear membrane disruption (black open arrow), mitochondria (m), degenerated mitochondria (dm), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), fusion of light vesicles (white open arrow), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), cell debris (*), cell lysis (**). 55
- Figure 6:** Ultrastructure of OPS vitrified *in vivo* ovine embryos at early blastocyst stage fixed 1h after thawing. Blastocysts were classified as grade 1 (A and B) and grade 2 (C and D) in semithin sections. Perivitelline space (PvS), microvilli (Mv), blastocoelic cavity (b), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), lipid droplets (L). 56
- Figure 7:** Evaluation of vitrified (OPS) sheep embryos after warming, previously submitted to different protocols of lipid reduction. Embryos were scored (1-4) according to the post warming expansion: without expansion (non exp) = 1, semi-expansion (semi-exp) = 2, expansion (exp) = 3 and expansion with an excellent blastocoele (exp+blast) = 4. Values with different letter within individual columns are significantly different ($p < 0.05$). Experiment 1: cytochalasin D (cyto-D, n=24), centrifugation (cent, n=25), centrifugation and cytochalasin D (cent+cyto-D, n=22), control (control, n= 27) groups. 64
- Figure 8:** Evaluation of vitrified (OPS) sheep embryos after warming, previously submitted to a protocol of lipid reduction using different concentrations of *trans*-10 *cis*-12 conjugated linoleic acid isomer (CLA; 25 and 50 μ M) supplemented to the culture medium. Embryos were scored (1-4) according to the post warming expansion: without expansion (non exp) = 1, semi-expansion (semi-exp) = 2, expansion (exp) = 3 and expansion with an excellent blastocoele (exp+blast) = 4. Values with different letter within individual columns are significantly different ($p < 0.05$). Experiment 2: control (n= 14), CLA25 (n=23) and CLA50 (n=18) groups. 66
- Figure 9:** Evaluation of vitrified (OPS) embryos after warming, previously submitted to different protocol of lipid reduction. Embryos were scored (1-4) according to the post warming expansion: without

expansion (non exp) = 1, semi-expansion (semi-exp) = 2, expansion (exp) = 3 and expansion with an excellent blastocoele (exp+blast) = 4. Values with different letter within individual columns are significantly different ($p < 0.01$). Experiment 3: control (control, n= 25), cytochalasin D (cyto-D, n=22), centrifugation and cytochalasin D (cent+cyto-D, n=20), 25 or 50 μ M trans-10 cis-12 conjugated linoleic acid isomer (CLA) added in the culture medium (CLA25, n=33) and (CLA50, n=18) groups.67

Figure 10: Ultrastructure of fresh sheep blastocysts produced in culture media supplemented with 25 μ M of *trans*-10 *cis*-12 linoleic acid isomer (CLA25F). Outer (A,C,E) and inner cells (B,D,F), cells. ZP pellucida (ZP), microvilli (Mv), perivitelline space (PvS), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light (LV), dense vesicles (DV), lipid droplets (L), fusion of lipid droplets with dense vesicles (white open arrow), fusion of light vesicles (black arrow head), Golgi complexes (G), nucleus (N), nucleolus (Nc), blastocoele cavity (Bc) 87

Figure 11: Ultrastructure of fresh *in vitro* produced sheep blastocysts (control fresh, CF). Outer (A,C) and inner cells (B,D), cells. Microvilli (Mv), perivitelline space (PvS); mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), disrupted nuclear membrane (white open arrow), ** cell debris cytosolic areas devoid of organelles (Cy) blastocoele cavity (Bc)..... 88

Figure 12: Ultrastructure of OPS vitrified sheep blastocysts produced in culture media supplemented with 25 μ M of *trans*-10 *cis*-12 linoleic acid isomer (CLA25V) and fixed 1h after warming. Perivitelline space (PvS), microvilli (Mv), blastocoele cavity (b), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), fusion of light vesicles (black arrow head), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), cytosolic areas devoid of organelles (Cy)..... 89

Figure 13: Ultrastructure of OPS vitrified *in vitro* produced sheep blastocysts (control vitrified, CV), fixed 1h after warming. Perivitelline space (PvS), microvilli (Mv), blastocoele cavity (b), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), *, cell lysis, ** cell debris, disrupted nuclear membrane (black arrow). 90

Table index

Table 1: Evaluation of morphological parameters and capacitation status (chlortetracycline staining-CTC) of fresh (F) and frozen thawed (FT) ovine semen (least squares means \pm standard error).....	34
Table 2: Effect of different maturation media (M1 and M2) and oocyte fertilization by fresh (F) or frozen–thawed (FT) spermatozoa on embryo production rates (least squares means \pm standard error and p-values).....	35
Table 3: Effect of different maturation media (M1 and M2) and oocyte fertilization by fresh (F) or frozen–thawed (FT) spermatozoa on post-thawed embryo (n=105) viability evaluated by intact and re-expanded blastocyst rates and embryo cell counts (least squares means \pm standard error).	36
Table 4: Effect of different protocols of lipid reduction on sheep embryo post warming evaluation (4 sessions, experiment 1).	64
Table 5: Effect of a protocol of lipid reduction using different concentrations of <i>trans</i> -10 <i>cis</i> -12 conjugated linoleic acid isomer (CLA) during sheep embryo culture on production rates (9 sessions, experiment 2).	65
Table 6: Percentual distribution of sheep embryo morphological quality after culture with different concentrations of <i>trans</i> -10 <i>cis</i> -12 conjugated linoleic acid isomer (CLA; 9 sessions, experiment 2).	65
Table 7: Effect of different concentrations of <i>trans</i> -10 <i>cis</i> -12 conjugated linoleic acid isomer (CLA) during sheep embryo culture on production rates (9 sessions, experiment 3).	66
Table 8: Production rates (6 sessions) of <i>in vitro</i> produced sheep embryos cultured with 25 μ M of <i>trans</i> -10 <i>cis</i> -12 linoleic acid isomer (CLA25 group) or without (control group).	80
Table 9: Percentual distribution of morphological quality (6 sessions) of <i>in vitro</i> produced sheep embryos cultured with 25 μ M of <i>trans</i> -10 <i>cis</i> -12 linoleic acid isomer (CLA25 group) or without (control group). .	80
Table 10: Post-warming survival (3 sessions) of <i>in vitro</i> produced sheep embryo cultured with 25 μ M of <i>trans</i> -10 <i>cis</i> -12 linoleic acid isomer (CLA25 group) or without (control group) and vitrified by open pulled straws (OPS).	81

List of abbreviations

Abbreviation	Unabbreviated	Abbreviation	Unabbreviated
μL	microliter	INIAV	National Institute of Agrarian and Veterinarian Research
AI	artificial insemination	INRB	Instituto Nacional dos Recursos Biológicos
BL	blastocysts	IVC	<i>in vitro</i> culture
BME	BME Amino Acids Solution	IVF	<i>in vitro</i> fertilization
BSA	bovine serum albumin	IVM	<i>in vitro</i> maturation
Ca	calcium	IVP	<i>in vitro</i> production
CLA	<i>trans</i> -10 <i>cis</i> -12 conjugated linoleic acid isomer	LH	luteinizing hormone
CO ₂	carbon dioxide	LN ₂	liquid nitrogen
COCs	cumulus oocyte complexes	LOPU	laparoscopic ovum pick-up
CTC	chlortetracycline	M199	tissue culture medium 199
D6	day 6	MEM	MEM Amino Acids Solution
D8	day 8	min	minutes
DMSO	dimethylsulphoxide	mm	millimeters
E2	estradiol	MOET	multiple ovulation and embryo transfer
EG	ethylene glycol	N ₂	nitrogen
EGF	epidermal growth factor	mM	miliMole
F	fresh	O ₂	oxygen
FCS	fetal calf serum	oFSH	ovine FSH
FF	follicular fluid	OPS	open pulled straws
fig.	figure	p.i.	post-insemination
FSH	follicle stimulating hormone	PBS	phosphate buffer saline
FT	frozen-tawed	PI	propidium iodide
GSH	glutathione	PPARs	peroxisome proliferator-activated receptors
h	hour	PUFA	polyunsaturated fatty acid
HA	hyaluronic acid	PVs	perivitelline space
HBP	hexosamine biosynthetic pathway	ROS	reactive oxygen species
HM	holding medium	SOF	synthetic oviductal fluid
hr	hour	SOFserum	fetal calf serum
ICBAS	Institute of Biomedical Sciences Abel Salazar	TCM	tissue culture medium
IETS	International Embryo Transfer Society	TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
im	intramuscular	UMIB	Unit for Biomedical Research

1 Preamble

Sheep production is one of the main livestock outputs in the Mediterranean area, being characterized by a large genetic diversity with several breeds of small ruminants (Gama, 2006). The importance of applying reproductive technologies is one of the cores of field action of the technicians working in Animal Science and Veterinary Medicine areas hereafter. In this context it is our opinion that progress in reproductive biotechnologies research is essential to guarantee the support of farmers, breeders associations and research centers.

Production and storage of sheep embryos is one of the new challenges that need to be appraised. This technology can be associated to other techniques as oestrus synchronization and superovulation, as well as semen technology, which have been applied more often, and should also include *in vitro* production (IVP) of embryos. In Portugal IVP of sheep embryos started in 2000 (Baptista *et al.*, 2002) and *in vivo* derived sheep embryos production has been routinely used with the foundation of the Portuguese Animal Germplasm Bank, established in 2005 (Pereira and Marques, 2008).

As will be discussed below, IVP of sheep embryo is still a demanding issue for researchers due to the inferior results when compared to other species and even more if cryopreservation is applied (Cognié *et al.*, 2004; Dattena *et al.*, 2004; Dalcin *et al.*, 2013). Therefore special efforts to develop new methods that could overcome the low cryotolerance of IVP sheep embryos should be addressed. Challenged by these constraints and motivated by the belief in the future need of knowledge in this area, we developed several experiments focused in the characterization of the effects of cryopreservation in *in vitro* produced sheep embryos and we delineate a strategy to reduce lipid content of IVP sheep embryos pointed as one of the causes of the low success in their cryopreservation. To develop this research work we joined some research teams working in this area, based in the Unit of Genetic Resources, Reproduction and Animal Breeding, of the National Institute of Agrarian and Veterinarian Research (INIAV), in Estação Zootécnica Nacional, Santarém, where the main part of the works took place, namely the steps of *in vitro* embryo production, semen technology, embryo cryopreservation and storage as well as other essential research steps. The source of oocytes was a regional slaughterhouse, Matreze, in Pedrogão Grande, and *in vivo* embryos derived from Merino sheep collected in the facilities of the Regional Agriculture Direction of Alentejo located in Herdade da Abóbada in Vila Nova de S. Bento, Serpa.

The need of fluorescence microscopy facilities led us to the Institute of Molecular Medicine, in the Faculty of Medicine of the University of Lisbon, where embryo cell counts and viability were performed.

PREAMBLE

The processing of embryos for electron microscopy occurred in the laboratory of Pathology of University of Évora and in the Department of Microscopy, Laboratory of Cell Biology of the Multidisciplinary Unit for Biomedical Research-UMIB, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, where images were obtained.

In a first approach we studied two methods of *in vitro* sheep embryo production using two different methods of *in vitro* maturation and also comparing fresh and frozen-thawed ram semen for *in vitro* fertilization. This contribution was published in the paper:

Romão R, Marques CC, Baptista MC, Vasques, MI, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Plancha C, Rodrigues P, Pereira RM. Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen. *Small Ruminant Research* 2013;110:36-41.

Afterwards we studied the effects of cryopreservation in sheep embryo, by highlighting the alterations that occur at ultrastructural level in IVP and their *in vivo* counterparts, either fresh or cryopreserved. At our knowledge these can be the first published results showing ultrastructural changes in IVP sheep embryos. The results of this experiment were compiled in a submitted paper:

Romão R, Bettencourt E, Pereira RMLN, Marques CC, Baptista MC, Barbas JP, Horta AEM, Oliveira E, Bettencourt C, Sousa M. Ultrastructural characterization of fresh and vitrified *in vitro* and *in vivo* produced sheep embryos. *Theriogenology* (submitted).

Postulating that delipidation of IVP of sheep embryos is essential for increasing their cryotolerance several experiments were performed using physical or chemical delipidation. Some of these techniques were already tried in other species being adapted to sheep IVP embryos for the first time. These experiments were included in another submitted paper:

Romão R, Marques CC, Baptista MC, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Pereira RM. Cryopreservation of *in vitro* produced sheep embryos: effect of different protocols of lipid reduction. *Theriogenology* (submitted).

Last but not least we studied the postulated positive effect of CLA in the ultrastructure of IVP sheep embryos enhancing cryotolerance. A paper was written with these results:

Romão R, Marques CC, Bettencourt E, Baptista MC, Barbas JP, Carolino N, Oliveira E, Sousa M, Pereira RMLN. Ultrastructure of *in vitro* produced sheep embryos: effect of *trans*-10 *cis*-12 conjugated linoleic acid (CLA) in fresh and cryopreserved blastocysts. *Animal* (submitted).

In the 6 years of this research, besides the published and submitted publications we had also the opportunity to present two communications in the scope of a National and an International Congresses, reporting some preliminary results of our data.

Knowing that much effort will still be needed to reach definitive methods to be used in cryopreservation of IVP sheep embryos, it is our believe that with the contributions of those that were involved in this work, we can add a small contribution to future fruitful discussion and research in small ruminant reproductive biotechnologies.

2 Introduction¹

In the last decade, the production of sheep embryos did not get an improvement as researchers would like to announce and its application is still lower than in other species (Isachenko *et al.*, 2003; Dalcin *et al.*, 2013), being embryo cryopreservation one of the trouble spots in this area. In fact, since the first steps in embryo cryopreservation, attempts have been made to understand the effect of freezing in sheep embryos (Willadsen *et al.*, 1976; Willadsen, 1977). It is expected that a successful cryopreservation of sheep embryos can push forward all other reproductive biotechnologies in this species as multiple ovulation and embryo transfer (MOET), artificial insemination (AI) or *in vitro* production of embryos. These associated techniques are still expensive and this fact limits its widespread.

In sheep, as in other species, there are two ways of obtaining embryos: *in vivo* derived or *in vitro* produced (IVP). *In vivo* embryo production consists in collecting them directly from the uterus either after natural service or after AI, while IVP involves oocyte collection from sheep ovaries followed by laboratorial maturation, fertilization and embryo culture. Advantages and uses of each technique have been previously discussed (Cognié *et al.*, 2003; Cognié *et al.*, 2004).

The highest lambing rates can be achieved after transfer of fresh *in vivo* derived embryos being reported results of 46.4-66.7% (Folch *et al.*, 2000), 67.8% (Martinez *et al.*, 2006), 73.3% (Bettencourt *et al.*, 2009b), 75% (Papadopoulos *et al.* 2002) and 81.2% (Dattena *et al.*, 2000). Conversely transference of fresh IVP embryos has lower results with lambing rates of 32.8% (Papadopoulos *et al.*, 2002), 37.5% (Martinez *et al.*, 2006) or 40% (Dattena *et al.*, 2000). Despite this variation, some authors referred no differences between surviving rates after transfer of fresh *in vivo* derived and IVP embryos (Dobrinsky, 2002).

Cryopreservation tries to preserve embryos to be maintained as genetic reserves or for easier application later on. However, the results are below the desirable. For instance, lambing rates reported after transfer of *in vivo* derived cryopreserved sheep embryos are of 32-36% (Folch *et al.*, 2000), 50% (Gibbons *et al.*, 2011), 58.3% (Bettencourt *et al.*, 2009b), 60% (Dattena *et al.*, 2001), 60.1-75.1% (Dattena *et al.*, 2004), 75% (Dattena *et al.*, 2000), whereas for IVP embryos are of about 21.7% (Dattena *et al.*, 2000), 19.4-23.8% (Dattena *et al.*, 2004) and 23-26.6% (Martinez *et al.*, 2006).

The analysis of these results highlights that the low cryotolerance is the main obstacle for using cryopreserved sheep embryos, especially the IVP embryos that present a much lower survival rate

¹ This part will be adapted to be submitted as a review paper.

compared to *in vivo* derived ones (Rizos *et al.*, 2002b). It has been demonstrated that IVP sheep embryos have a slower developmental rate with detrimental effects in placentation and higher fetal loss, leading to a lower lambing rate (Tveden-Nyborg *et al.*, 2005). An enhanced efficiency would increase the number of viable produced embryos per ewe making more feasible its use. For example, Folch *et al.* (2000) referred 4.5 lambs produced by ewe using MOET and AI followed by cryopreservation of embryos. Also the use of cryopreserved embryos can facilitate the work planning process, eliminating distance limitations and reducing sanitary risks (Folch *et al.*, 2000). In fact, the sanitary quality of *in vitro* produced embryos can theoretically be better controlled than those obtained *in vivo* (Guerin *et al.*, 2000).

In the last 15 years, researchers have tried to understand the reason of the low cryotolerance of IVP embryos, searching for new methods capable of improved results in this species. Sheep cryopreserved embryos present ultrastructural damage (Bettencourt *et al.*, 2009) clearly expressing the cryodamage of cells during the process of freezing and thawing. Cryopreserved embryos show less microvilli and lower mitochondria activity than fresh ones (Cocero *et al.*, 2002; Bettencourt *et al.*, 2009). The lower cryotolerance of IVP blastocysts can be related to the excessive accumulation of lipids (Thompson *et al.*, 1995; Dattena *et al.*, 2000; Rizos *et al.*, 2002b; Pereira *et al.*, 2007; Pereira and Marques, 2008) and these accumulation can be favored by their culture in serum containing media (Cho *et al.*, 2002; Abe and Hoshi, 2003; Pereira *et al.*, 2007) but also by the type and concentration of cryoprotectant used as well as the freezing protocol (Cocero *et al.*, 2002). No studies have been done that can explain the different alterations induced by cryopreservation in IVP embryos at ultrastructural level.

2.1 Application

On the behalf of developing new and improved reproductive assisted techniques, mainly by research teams, there has been for long an interest in sheep embryo model research as confirmed by the birth of the first IVP lambs in 1991 (Czlonkowska *et al.*, 1991; Pugh *et al.*, 1991) and the first cloned animal (a lamb) by nuclear transfer in 1997 (Wilmut *et al.*, 1997). Beyond that, great interest exists in developing embryo reproductive technology to be used commercially or in routine programs for conservation of species and breeds all around the world.

Food and Agriculture Organization (FAO) has established the minimum number of individuals in each population so they can have a classification of endangered (Henson, 1992; FAO, 1998). This classification is the base for choosing the target breeds or species to be urgently preserved. Also following the guidelines of Rio de Janeiro convention, in 1992, it was established that all countries should have a plan for the

INTRODUCTION

conservation of autochthon genetic resources, also recognizing their qualities in what concerns the adaptation to local conditions as well as their potential use in agricultural niches of production (Båge, 2003).

One of the best ways of preserving the animal genetic resources of endangered populations is through the creation of germplasm banks in which the biological material can be stored for decades, as cryopreservation, at actual knowledge, is a reliable way of long-term conservation of genetic resources (Fogarty *et al.*, 2000; Yao *et al.*, 2012). Besides, one of the current alternative strategies for maintenance of some breeds or strains is through embryo cryopreservation (Amstislavskya and Trukshinb, 2010), now routinely used in mouse strains for long-term colony maintenance, with the advantages of saving costs, readiness in distant transport, health guarantee and also for preventing genetic drift occurrence (Woods *et al.*, 2004; Mochida *et al.*, 2013).

In other perspective, germplasm exchanges are nowadays crucial (Thibier, 2011), being extremely useful in improving genetic upgrading (Gibbons and Cueto, 2011). Also embryo transfer can have a major role in reducing or eliminating some transmissible diseases in germplasm livestock changes (Stringfellow and Givens, 2000; Thibier and Guérin, 2000; Cognié and Baril, 2002; Pereira and Marques, 2008). For commercial proposes embryo transfer should be chosen for those reasons in which other less expensive techniques are not as advantageous. For example Cognié and Baril (2002) estimated a 10 times higher cost when comparing embryo transfer (either using *in vivo* or *in vitro* embryos) with artificial insemination and this represents an elevated cost attending the economic value of the animal species (Cognié and Baril, 2002).

Based on International Embryo Transfer Society (IETS) data, a decade ago, Thibier (2004) reported 6674 fresh and 2907 frozen/thawed sheep embryos transferred in the world in 2003 and, for the year of 2012, Perry (2013) referred 8124 fresh and 6134 frozen/thawed transferred embryos, mainly represented by Australia, South America and South Africa. This numbers, although considered underestimated because of difficulty in retrieving data, show an evolution of the use of this reproductive biotechnology and, in our opinion, reflects the demands of an emerging global market in this area.

In sheep, as in other species, several methods can be used for *ex situ* conservation of genetic resources and attention in reproductive cells has been focused in oocyte, spermatozoa, zygote or embryo cryopreservation (Mullen and Fahy, 2012; Mara *et al.*, 2013). In parallel, in the last years, research has been conducted in other methods as ovary, testicle (Arav *et al.*, 2005, Faustino *et al.*, 2010; Merdassi *et al.*, 2011; Mara *et al.*, 2013), and somatic cells cryopreservation (McCreath *et al.*, 2000; Liu *et al.*, 2010) as well as in

new methods in embryo technology as somatic cell nucleus transfer and transgenesis (McCreath *et al.*, 2000; Betteridge, 2006).

Having the background of other species we can mention that, for example, in the United States, in 2011, 79% of all bovine collected embryos were later cryopreserved (Hasler, 2012). In sheep, cryopreservation of embryos is a crucial developing technique for commercial application and it is our idea that, when better results after the cryopreservation of embryos have been attended, it will be the dominant target for sheep embryo production and also using IVP embryos because of the possibilities that can be provided.

2.2 *In vitro* embryo production

In vitro embryo production simulates the natural development of an embryo and comprises the phases of oocyte maturation, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of embryos. In these steps the last one is the critical in terms of determining the blastocyst yield (Lonergan and Fair, 2014). In terms of lambing rate, IVP embryo survival is 25% lower than for *in vivo* derived ones mainly due to an increased embryo loss at day 30-40 of gestation (Cognié *et al.*, 2004). Betts and King (2001) have discussed the mechanisms of preimplantation and cell death referring the importance of knowing the tools and conditions that can promote better results in IVP. Moreover Betts and Madan (2008) referred the importance of permanent embryo demise that occurs in the first week of development in the outcomes of assisted reproductive technologies.

In parallel with other species, IVP technology reduces the rate of produced embryos and leads to inferior embryo survival when compared to *in vivo* derived embryos, as previously discussed (Cognié and Baril, 2002; Tominaga, 2004; Pereira and Marques, 2008; Blanco *et al.*, 2011; Lonergan and Fair, 2014). Cocero *et al.* (2011) obtained 34.6% (day 7) or 40.4% (day 8, D8) blastocysts produced from sheep abattoir derived oocytes and Cognié *et al.* (2003) referred 25% D8 blastocyst yield.

In vitro sheep embryo production is not yet a controlled and totally defined technique once that has been associated with the “large offspring syndrome” (Thompson *et al.*, 1995; Holm *et al.*, 1996) and other not well understood problems (Shirazi *et al.*, 2009), also reported in other species (Farin *et al.*, 2001; Lazzari *et al.*, 2002). These abnormalities seem to be promoted in very early developmental stages, such as IVM or IVF, irrespectively of the subsequent *in vivo* or *in vitro* culture treatment (Holm *et al.*, 1996; Galli and Lazzari, 2008). Apparently the use of serum supplementation and co-culture can be pointed as a justification (Galli and Lazzari, 2008). Also these problems could be extended following birth as Çörekçi and Yilmaz (2004) found higher growing rates until weaning age in lambs born from IVP. It is well established that IVP embryos

INTRODUCTION

have an altered morphology when compared with *in vivo* derived counterparts (Crosier *et al.*, 2000). It has also been observed an increase in the lipid content of IVP compared to *in vivo* derived embryos regardless of the composition of the medium of culture. Blastocyst's differences depending on culture medium used were identified (Thompson *et al.*, 1995; Crosier *et al.*, 2001; Pereira *et al.*, 2007). These changes have also been correlated to the lower cryotolerance of IVP embryos as stated before.

Several studies have tried to establish the most successful laboratory method of IVP including IVM, IVF and IVC of embryos since all these steps are crucial for good results.

2.2.1 Collection of oocytes

The physiological constraints on sheep uterus access, mainly by the peculiar anatomy of the cervix, have reduced the chances of an easy collection of oocytes and embryos, although interest in new techniques (Gusmão, 2011), which also could improve welfare issues in future, exists. There are several techniques of oocyte collection namely by follicular aspiration or slicing in abattoir derived ovaries (Wani *et al.*, 2000; Zeinoaldini *et al.*, 2013). Better quality of cumulus oocyte complexes (COCs) and easy application have been associated with the later technique (Wani *et al.*, 2000; Wani, 2002). Also the number of harvested oocytes collected after slicing is higher (Amiridis and Cseh, 2012). However slicing is a very time consuming technique with the associated loss of oocyte viability during the process (Machado *et al.*, 2012). On the other hand, sheep ovum pick up-systems are applied in living animals using minimal invasive procedures such as laparoscopic ovum pick-up (LOPU) technique guided by laparoscopy (Baldassarre *et al.*, 1996, 2002; Rodriguez *et al.*, 2006; Cox and Alfaro, 2007; Gibbons *et al.*, 2008). These procedures, when used *in vivo*, are usually associated with follicular stimulation treatments (Gibbons *et al.*, 2007) in order to raise the number of collected COCs (Mermillod *et al.*, 2006; Crocomo *et al.*, 2012) and can allow better maturation, fertilization and *in vitro* development capacities (O'Brien *et al.*, 1997; Cocero *et al.*, 2011). According to Stangl *et al.* (1999) and Bari *et al.* (2001) it is possible to repeatedly collect oocytes by this approach without reducing subsequent recovery rates. Moreover, development of *in vivo* techniques of oocyte collection is important because, when derived from slaughtered animals, oocytes usually cannot be used in genetic programs due to the unknown sanitary and even genetic status, being applied mainly for research purposes (Gibbons *et al.*, 2008). However a safe animal identification system may overcome these issues.

Oocyte quality seems to be the key limiting factor to achieve high embryo production ratios and quality (Dieleman *et al.*, 2002; Cognié *et al.*, 2003; Blanco *et al.*, 2011). Embryo production depends also in oocyte competence to complete meiosis which is affected by follicle size and quality (Cognié and Baril, 2002). It is known that pre-pubertal oocytes have an inferior developmental potential than those collected from adults (O'Brien *et al.*, 1996, 1997). Nevertheless the expected potential in future results using juvenile oocyte collection, namely the decreasing to half of the generation interval in genetic programs, must be considered (Amiridis and Cseh, 2012).

2.2.2 *In vitro* maturation (IVM)

Collected oocytes must undergo cytoplasmic and nuclear maturation (reaching metaphase II stage) prior to fertilization. Once outside the follicle, spontaneous meiotic resumption is the beginning of oocyte *in vitro* maturation (Mermillod *et al.*, 2006). This is usually performed in an incubator at 38-39°C in a humidified atmosphere with 5% CO₂ concentration (Pereira *et al.*, 2009; Amiridis and Cseh, 2012). The widely used medium for IVM is Tissue Culture Medium (TCM) supplemented with bicarbonate, pyruvate, luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E2) and with 10% fetal calf serum (Cognié *et al.*, 2004; Cocero *et al.*, 2011). Gonadotrophins seem to play a central role in the process as well as serum (Accardo *et al.*, 2004; Wani *et al.*, 2012).

Nevertheless other media and supplementation can be also used. For example, Shabankareh *et al.* (2011) found good results using human menopausal serum and Birler *et al.* (2001) showed that, in sheep, synthetic oviductal fluid (SOF) medium improves the rate of cleavage compared to TCM199 medium. Guler *et al.* (2000) presented higher results adding follicular fluid (FF) to maturation medium or using epidermal growth factor (EGF) conjugated with FSH and E2. Also Cotterill *et al.* (2012) and Zhou *et al.* (2008) showed the positive effect of EGF or other EGF-like ligands in COCs during IVM and Alabart *et al.* (2000), Cocero *et al.* (2011) and Wani *et al.* (2012) with EGF and cysteamine.

Despite atmosphere control of O₂ concentration in IVM, the whole process of IVP is stressful because of the formation of reactive oxygen species (ROS) that promote cellular and nuclear damage (Deleuze and Goudet, 2010). Cumulus cells are important to the success of *in vitro* fertilization (Van Soom *et al.*, 2002; Shirazi *et al.*, 2007; Bogliolo *et al.*, 2007) but it is known by studies in bovine that these cells also undergo apoptosis during the IVM process (Ikeda *et al.*, 2003). In sheep these damage can be reduced with supplementation with the previously referred antioxidants as cysteamine, combined with growth factors, or

INTRODUCTION

using serum as font of albumin that balances osmolarity and acts as a free radical scavenger (Thompson, 2000) consequently improving maturation rates and subsequent IVP rates (Wani *et al.*, 2012).

2.2.3 *In vitro* fertilization

The fertilization of matured oocytes can be performed with frozen or fresh semen. Although some authors argue that early embryo development is not influenced by the method of semen preservation (Hollinshead *et al.*, 2004), fresh semen can improve embryo production rates and quality (Romão *et al.*, 2013), eventually because freezing and thawing disrupts the stability of ram sperm chromatin, reducing its fertilization efficiency (Peris *et al.*, 2004; Khalifa and Lymberopoulos, 2013).

Cryopreserved semen is advantageous since it can be easily available for routine use and can be stored in seasonal propitious period. However when a proven fertility ram semen is available it should be used in fresh either.

Fertilization is performed with previously capacitated spermatozoa usually using media containing heparin or sheep serum (Cognié *et al.*, 2004), that causes Ca^{++} income into the acrosome and thus capacitation and acrosome reaction (Freitas *et al.*, 2007). The best semen fraction is usually achieved by a Percoll gradient centrifugation (Cognié *et al.*, 2004) or swim-up (Suttiyotin and Thwaites, 1993; Mermillod *et al.*, 2006) prior to fertilization. It is stated that both sperm preparation methods allow to select a sperm population with a low percentage of apoptotic spermatozoa (Ricci *et al.*, 2009).

2.2.4 *In vitro* culture

The goal of IVC is to simulate the events that occur in the oviduct so that putative zygotes obtained after fertilization undergo optimal development into blastocysts. For this reason culture can be performed *in vivo* using surrogate oviducts (Lazzari *et al.*, 2010). Usually embryo IVC is achieved in controlled atmosphere, at 38.5-39°C with 5%O₂, 5% CO₂ and 90% N₂ (Cognié *et al.*, 2004). The culture medium is usually composed of SOF supplemented with amino acids, bovine serum albumin (BSA) and/or serum (Birler *et al.*, 2001; Pereira *et al.*, 2009; Amiridis and Cseh, 2012; Romão *et al.*, 2013) although co-culture with oviduct cells or fetal fibroblasts was also reported (Bernardi, 2005). During IVC it is important to keep low concentration of oxygen preventing oxidation (see Harvey, 2007, for details) and the deleterious effects of ROS; this is also the reason of adding low molecular weight thiol compounds in IVM and IVC like cysteamine, β-mercaptoethanol, cystine and cysteine that increase the synthesis of glutathione (GSH), which in turn reduces the oxidative stress (De *et al.*, 2011). At this stage of embryo development, the generation of

energy is provided mainly by the Krebs cycle and oxidative phosphorylation. Therefore substrates as pyruvate, amino acids, lactate and fatty acids are important to embryo development and mitochondria plays a central role in metabolic events. Oxygen consumption is lower before the morula stage, having an increasing during compaction with the higher glucose consumption via glycolysis after blastocysts formation (Leese, 2012). Providing the best IVC conditions is of primordial importance for embryo competence acquisition to complete a term pregnancy after transfer.

2.3 Cryopreservation of sheep embryos

The science of cryobiology aims to preserve cells at low temperatures (-196°C), intending to avoid the negative effects of the process which can preclude the main objective. The survival of cells depends on both the freezing and the warming processes. Cryopreservation of sheep embryos is a difficult task and practical results are not as encouraging as in cattle mainly due to the reduced embryo cryotolerance in this species.

Two major methods have been used to preserve embryos: slow freezing and vitrification. The difference between them is the velocity rate of freezing. While slow freezing tries to differentiate the cooling rate in sequential steps of freezing, vitrification increases the freezing velocity by reducing volume, thus minimizing ice formation (Gao and Critser, 2000; Arav 2014). Slow freezing is currently practiced in programmable freezers and has the advantage of using low concentrations of cryoprotectants, but their ability to prevent ice-crystal formation at low concentrations is limited (Pereira and Marques, 2008). Vitrification is considered an important methodology for long-term storage of embryos in a glass-like amorphous vitreous state without the formation of ice crystals (Vajta and Nagy, 2006; Kim *et al.*, 2012). Several methods using this technique are presently available: conventional vitrification or adaptations of the last one, such as vitrification in open pulled straws (OPS, Vajta *et al.*, 1998) or more recently in other devices such as cryotop (Kuwayama, 2007) and paper containers (Kim *et al.*, 2012) among others.

As previous described, in all the species *in vitro* produced embryos presented a higher sensitivity to cryopreservation than *in vivo* ones. Several reasons have been indicated to justify this IVP cryosensitivity, mainly cellular (Pereira *et al.*, 2007; Rizos *et al.*, 2002b), metabolic (Khurana and Niemann, 2000; Farin *et al.*, 2001) and biochemical (Massip *et al.*, 1995) changes. At ultrastructural level, *in vitro* produced blastocysts exhibited less microvilli and a less extensive network of intercellular junctions, specifically an apparent lack of desmosomal junctions, a higher incidence of cellular debris and a higher number of lipid droplets than their *in vivo* counterparts (Farin *et al.*, 2001; Rizos *et al.*, 2002a). These differences could

INTRODUCTION

certainly contribute to the observed lower cryotolerance of *in vitro* produced embryos (Massip *et al.*, 1995; Ohboshi *et al.*, 1998; Cognié and Baril, 2002).

Although it seems that very low temperatures will disrupt cell components, the most complicated freezing window is between -15°C and -60°C followed by intracellular ice formation, between -5°C and -15°C (Gao and Critser, 2000), whereas between -50°C and -150°C the risks are fracture of the zona pellucida or cytoplasm (Rall and Meyer, 1989). Below -150°C the risks in embryo damage are lower (Vajta and Nagy, 2006). In embryo cells with high lipid content, as in sheep, the lipid drops can combine with parts of the cytoskeleton, as well as membranes, organelles and other structures of the cytoplasm. When these embryos are cooled from 15°C until -5°C this association can be related to irreversible and fatal structural damage occurrence, mostly when the traditional freezing methods are used (Dinnyes *et al.*, 2006; Vajta and Nagy, 2006). Most of these injuries seem to be related with cell lipid content and specifically to the membrane's saturated-to unsaturated fatty-acid ratio (Arav *et al.*, 2000; Arav and Zvi, 2008). During exposure to sub-physiological temperatures, the membrane goes through a lipid phase transition with solidification of lamellar lipids, such as saturated fatty acids. The low-temperature-fluid lipids, such as unsaturated fatty acids, which in this case are non-lamellar, then separate and form a new non-lamellar structure, such a II hexagonal structure which interferes with membrane function and leads to ion leakage and cell death (Arav and Zvi, 2008). Different strategies can be implemented to minimize cryodamage (Arav and Zvi, 2008; Pereira and Marques, 2008).

Both approaches for cryopreservation, slow freezing and vitrification, have been applied using *in vivo* derived or IVP embryos in sheep (Baril *et al.*, 2001; Dattena *et al.*, 2004; Bettencourt *et al.*, 2009) or in cattle (Assumpção *et al.*, 2008). In *in vivo* derived sheep embryos no differences were observed in lambing or embryo survival rates after transfer of embryos cryopreserved using slow freezing, conventional vitrification or vitrification in OPS (Bettencourt *et al.*, 2009).

Although the achieved results by each method can be relevant, the cost and application in routine field is also of primordial importance to spread its use (Baril *et al.*, 2001; Dattena *et al.*, 2004).

2.3.1 Slow freezing

This method tries to control the descending cooling event in several steps, regulating extracellular and intracellular water exchange, with a balance between ice crystal formation and structural and osmotic damage, as well as cryoprotectant toxicity (Vajta and Nagy, 2006; Gajda and Smorag, 2009). The method is

possible using as referred programmable freezers with a rapid cooling until -6 to -7°C, when ice formation is induced (seeding), and then a lower rate of cooling (0.3 to 1°C/min) causing freezing of extracellular ice and increasing the concentration of extracellular solution and cellular dehydration. At -30°C, straws are then plunged in liquid nitrogen (Woods *et al.*, 2004; Saragusty and Arav, 2011).

In spite of still being used by many teams in the world it is argued that slow freezing has a limited future in embryology and according to Vajta and Nagy (2006) “the rate of advancement in oocyte and embryo cryopreservation will depend on the rate by which embryologists and decision-makers adopt the new approaches”.

2.3.2 Vitrification

Vitrification was found to be interesting in embryo cryopreservation because it reduces cryoinjuries caused by ice formation (Rall and Fahy, 1985). In fact, vitrification is based on embryo manipulation into different carrier tools applied to minimise the volume and to submerge the sample quickly into liquid nitrogen, allowing an ultra-fast freezing speed, which avoids ice crystals formation, thus eliminating its deleterious effects in the cell (Dalcin and Lucci, 2010). This technique combines the use of small volumes with high concentration of two or more cryoprotectants (Liebermann *et al.*, 2002) and it can be more adapted to IVP embryos (Vajta *et al.*, 1998; Massip, 2001; Kasai and Mukaida, 2004).

According to several authors, the slow freezing techniques are time consuming and laborious (Assumpção *et al.*, 2008) whereas vitrification is simple, rapid and inexpensive (Vajta *et al.*, 1998; Gupta and Lee, 2010; Saragusty and Arav, 2011). Moreover, no publications have demonstrated that results obtained by vitrification were significantly worse than those obtained by slow freezing (Vajta and Nagy, 2006). Concerns in safety of vitrification related to transmissible diseases are partially justified but new methods have been developed to avoid these constraints (Yu *et al.*, 2010).

Several authors described good results with this cryopreservation technique in sheep. For example, Baril *et al.* (2001) and Bettencourt *et al.* (2009b) achieved good field results (50-60% lambing rate) with vitrification of *in vivo* derived sheep embryos even with direct transfer of cryopreserved embryos and Folch *et al.* (2000) referred lambing rates of 32 to 36%, also in *in vivo* derived embryos.

In IVP embryos, Ptak *et al.* (1999) found no differences in pregnancy rates between fresh and vitrified transferred embryos (47 vs 42%). However they achieved significantly differences in lambing rates (41 vs 23%), showing that vitrification of IVP embryos is not yet an optimized technique.

INTRODUCTION

The success of vitrification depends on the stage at which embryos are cryopreserved apart from the method used. In fact there are different levels of sensitivity to low temperatures, depending on the stage of embryo development (Balasubramanian and Rho, 2006). Garcia-Garcia *et al.* (2005, 2006) and Shirazi *et al.* (2010) showed that cryotolerance of IVP embryos to conventional slow freezing or vitrification increased as the developmental stage of embryos progressed perhaps due to the higher cryotolerance of blastocysts when compared with early developmental embryos. In fact, Garcia-Garcia *et al.* (2006), using slow freezing, described similar rates of viability between fresh or frozen-thawed embryos (92.5 vs 83.7%) frozen at the blastocyst stage.

2.3.3 Vitrification in OPS

In order to achieve a higher rate of temperature reduction the OPS method uses thinner superfine straws that have the end of the straw with half the diameter of a conventional one. This characteristic enables filling by capillary action with a volume of approximately 1µL, different from the conventional straw that contains 5µL (Vajta *et al.*, 1998; Vajta and Nagy, 2006). So, it can allow freezing rates of 20000°C/min (Massip, 2001), i.e., 10 times higher than in 0.25mL straws and overcome some problems of vitrification as toxicity of cryoprotectants, and difficulties in the permeability of membranes that can lead to intracellular ice formation and osmotic over-swelling (Kasai and Mukaida, 2004). Allowing higher speed of freezing, this type of vitrification is helpful in preventing embryo and zona pellucida fracture that occurs at low temperatures, especially with appropriate adjustments of warming parameters (Kuwayama, 2007). The method has been introduced by Vajta *et al.* (1998) and, since then, it has been successfully used in several animal species. It is a robust and feasible method for animal embryo vitrification, more than other new, but delicate, techniques (Vajta and Nagy, 2006).

Vitrification by OPS is so effective that results in lambing rate of *in vivo* derived sheep embryos vitrified by this method are persuasive. Green *et al.* (2009) reported higher pregnancy rates when using vitrification by OPS and direct transference, thus enhancing the field application of this technique. Nevertheless, these good results with OPS vitrification cannot avoid the reduced cryotolerance of IVP embryos. Dattena *et al.* (2001) achieved lambing rates of 60% for *in vivo* produced embryos vitrified in OPS while only 24% for IVP.

2.3.4 Cryoprotectants

The role of these substances is to reduce damage to cryopreserved embryos, minimizing ice formation. They usually are classified in permeable and non-permeable cryoprotectants, being also important in osmotic dehydration (Vajta and Nagy, 2006; Pereira and Marques, 2008).

The negative effects of cryoprotectants are related to their osmotic and toxic effects, closely dependents of the time of exposure and the concentration used (Massip, 2001; Dinnyes *et al.*, 2006; Arav, 2014). The intent to reduce cryoprotectants to a minimum is a goal that has been tried in both slow freezing and vitrification procedures to minimize their deleterious effects. According to Liebermann *et al.* (2002) a balance between the maximization of cooling rate and the minimization of cryoprotectant concentration is the key point for a successful cryopreservation. Mainly in vitrification, where it is necessary to deal with higher concentrations of cryoprotectants, one of the strategies is to use more than one. This strategy reduces individual toxicity of cryoprotectants and also permits adding them in a stepwise equilibration (two or three steps), with increasing concentration or after cooling from 4°C to subzero temperatures when their toxicity is lower (Massip, 2001; Woods *et al.*, 2004; Vajta and Nagy, 2006).

Ethylene glycol (EG) is a frequent choice as a permeable cryoprotectant but others can be used like acetamide, glycerol, raffinose and dimethylsulphoxide (DMSO) in several combinations. For non-permeable cryoprotectants mono- and disaccharides including sucrose, trehalose, glucose and galactose can be cited, being sucrose the most used (Vajta and Nagy, 2006). The latter molecules, with large molecular weights, do not penetrate the cell membrane, but they can significantly reduce the amount of cryoprotectant required as well as the toxicity of EG by decreasing the concentration required to achieve a successful cryopreservation (Liebermann *et al.*, 2002). Other substitute substances like polymers and proteins (e.g. polyvinylpyrrolidone, polyethylene glycol and Ficoll), not being essential, have been tried to replace the former indicated components by minimizing toxic effects (Liebermann *et al.*, 2002; Kasai and Mukaida, 2004).

Different embryos cryosurvival rates were found among cryoprotectants with higher results for EG than glycerol (Martínez and Matkovic, 1998; Cocero *et al.*, 1996, 2002). According to Cocero *et al.* (2002) EG protects embryos membranes and cytoplasmic structures from cryoinjury but Kartberg *et al.* (2008) have also reported good results of DMSO in protecting embryo membranes.

INTRODUCTION

2.3.5 Warming of thawed embryos

Embryo thawing should be performed also in order to reduce cryoinjuries, when passing critical temperatures. In embryos cryopreserved by slow freezing the best method depends on the specific protocol and cryoprotectant used. Briefly, if the freezing method finished at temperatures of -30 to -40°C, a moderately rapid warming (200 to 350°C/min) is required to maximize the survival rate but if freezing reached -60°C or less, warming rate should be slow, in the range of 25°C/min (Gupta and Lee, 2010) although injuries were reported (Hochi *et al.*, 1996).

Warming of vitrified embryos is usually performed directly into a solution at body temperature although it can be advisable to wait 1-3 sec in air to avoid fracture damage (Vajta and Nagy, 2006). Vitrified embryos need to be warmed ultra-rapidly in the presence of non-permeating cryoprotectants (usually sucrose) to dilute and remove the very high levels of intracellular permeating cryoprotectants (Gupta and Lee, 2010); this can lead to changes in dynamics of water in the cells that can cause embryo injury caused by osmotic over-shrinkage (Kasai and Mukaida, 2004; Woods *et al.*, 2004).

Regardless of the method used for embryo warming, and transference after observation of re-expansion, Isachenko *et al.* (2003) Dattena *et al.* (2004) and Green *et al.*, (2009) obtained promising results for field use after direct transfer of vitrified sheep embryos. These authors reported that the interval between thawing and transference has a great influence on embryo subsequent viability and valorize direct transfer use for field conditions.

2.4 Methods for improving cryopreservation of sheep embryos

Different strategies to increase cryotolerance of embryos in sheep and in other species were developed mainly by improving embryo cryopreservation procedures, changing the composition of IVC media (Dattena *et al.*, 2007) or through the decrease of their lipid content (Arav, 2014; Prates *et al.*, 2014). As referred, methods of ultra-rapid vitrification have been developed to further increase freezing velocity. However, the ideal device and combination of cryoprotectants has not yet been achieved. Therefore, this technique continues to be a challenge in embryo cryopreservation (Gajda and Smorag, 2009). Some examples of devices are glass micropipette, solid surface vitrification, cryoloop, microdrop, cryotop, cryo-tip, electron microscopy grids or nylon mesh (Kong *et al.*, 2000; Kelly *et al.*, 2005; Kuwayama, 2005, 2007; Gajda and Smorag, 2009; Gupta and Lee, 2010; Gibbons *et al.*, 2011). Also attempts were experienced in IVC conditions as it was argued that the effect of changing culture conditions can be seen only after embryo

cryopreservation and warming. For example, Dattena *et al.* (2007) confirmed higher cryosurvival results when including BSA and Hyaluronan during IVC and Gad *et al.* (2012) realized that changing culture conditions lead to adaptations of embryos, induced by different gene expression, which was responsible for different developmental ability.

Difference in species' cryosensitivity of embryos is responsible for different approaches in their cryopreservation. As indicated before, one of the major concerns in embryo cryopreservation is its lipid content that can hamper cryopreservation (Thompson *et al.*, 1995; Dattena *et al.*, 2000; Tsujii *et al.* 2001; Rizos *et al.*, 2002b; Steel and Hasler, 2004; Seidel Jr., 2006; Pereira *et al.*, 2007; Pereira and Marques, 2008) as it happens in species as sheep or pigs. This phenomenon is more pronounced in IVP embryos (Leibo *et al.*, 1995, 1996) and in those produced in serum containing media (Thompson *et al.*, 1995; Cho *et al.*, 2002; Abe and Hoshi, 2003; Abe *et al.*, 2004; Pereira *et al.*, 2007). Serum is useful in oocyte and embryo culture as a source of albumin that balances the osmolarity, acting as a free radical scavenger with an additional important nutritive role (Thompson, 2000). However, the fatty acids and lipoproteins of the serum seem to be the source of embryos' cytoplasmic lipids, hampering embryo quality (Abe *et al.*, 2004; Lapa *et al.*, 2005), albeit the perturbations induced by the presence of serum in sheep embryo culture are higher before rather than after compaction (Rooke *et al.*, 2007).

On the other hand, embryo lipid content effect on chilling sensitivity is not totally elucidated at the moment. However it seems that lipid droplets interact directly with the intermediate filaments of the cytoskeleton and changes within these organelles during the cryopreservation process may lead to irreversible damages in the cytoskeleton (Pereira *et al.*, 2008; Zehmer *et al.*, 2009). Also it has been observed that cryopreserved embryos have ultrastructural changes that are visible as degenerated cells, disruption of cell membranes, and mitochondrial injuries, mainly in poor quality embryos (Dobrinisky, 1996; Cocero *et al.*, 2002; Bettencourt *et al.*, 2009). Damages in mitochondria are clear and reported by other authors before (Vajta *et al.*, 1997; Cocero *et al.*, 2002; Cuello *et al.*, 2007; Dalcin *et al.*, 2013) and may be used as reliable sign of cellular damage (Bettencourt *et al.*, 2009, 2014). Mitochondrial changes have been observed in poor quality and IVP embryos and were associated with culture in serum medium (Shamsuddin *et al.*, 1994; Abe *et al.*, 1999). Several changes have been reported, namely a reduced total number and an increase in the proportion of immature mitochondria (Abe *et al.*, 2002). These changes have been also associated with inefficient lipid metabolism and poor quality embryos that compromises ATP production (Doorland *et al.*, 1997; Crosier *et al.*, 2000; Abe *et al.*, 2002, 2004; Gad *et al.*, 2012). Also these

INTRODUCTION

mitochondrial changes have been related with the presence of cytoplasmic vesicles and lipid drops and fewer lysosome-like vesicles (Shamsuddin *et al.*, 1994; Abe *et al.*, 1999).

Due to this negative effect of embryo lipid content and composition in chilling sensitivity, attempts have been made to reduce its amount in IVP embryos either by chemical or by physical approaches. Nevertheless lipids are important in the embryo cell metabolism as a source of energy and are also essential for membrane formation and as intracellular messengers (McEvoy *et al.*, 2000; Tsujii *et al.*, 2001). Thus these strategies should be carefully evaluated. For instance in cattle, lipolytic agents or chemicals delipidators have been successfully applied increasing cryotolerance of vitrified embryos (phenazine ethosulfate, Barceló-Fimbres and Seidel Jr., 2007; *trans*-10 *cis*-12 conjugated linoleic acid, Pereira *et al.*, 2007, 2008; forskolin, Sanches *et al.*, 2013). These chemicals that regulate metabolism were used to reduce embryo lipid content, inducing smaller lipid droplets and fat indexes, thus improving embryonic cryosurvival (Pereira *et al.*, 2007, 2008; Accorsi 2008). Likewise Nagashima *et al.* (1994) demonstrated that the high lipid content of pig embryos was responsible for their higher chilling sensitivity and that delipidated embryos by centrifugation and microaspiration of polarized lipids became more tolerant to chilling. The lipid content was also pointed as responsible for the chilling and freezing sensitivity of IVP cattle embryos and when lipid droplets were displaced by centrifugation (mechanical delipidation) their cryosurvival was improved (Leibo *et al.*, 1995; Ushijima *et al.*, 1999; Diez *et al.*, 2001).

Other approaches to improve embryo cryosurvival have been investigated. Cytoskeleton relaxant/stabilizers as cytochalasin B or D were previously used during cattle and pig embryos vitrification to prevent cellular disruption, specifically to the embryonic cytoskeleton during and after cryopreservation (Dobrinsky *et al.*, 2000; Dobrinsky, 2002; Franco and Hansen, 2006) with either no benefits (Mezzalira *et al.*, 2002) or with positive results (Dobrinsky *et al.*, 1997; Tominaga *et al.*, 2000; Franco and Hansen, 2006). These molecules induce microfilament depolymerization before and during vitrification (Dobrinsky, 1996), acting as microfilaments inhibitor thus preventing actin polymerization and making the plasmatic membrane and cytoskeleton more elastic. Therefore embryo microfilaments and cytoskeletal architecture are not destroyed during micromanipulation, but also become more resistant to the osmotic stress induced by the exposure and removal of cryoprotectants during the vitrification/warming process (Dobrinsky *et al.*, 2000; Franco and Hansen, 2006). Dobrinsky *et al.* (2001) have obtained an 82% birth rate in pig vitrified embryos using cytochalasin B. Moreover the association of two strategies, cytoskeleton relaxants and centrifugation,

were successfully attempted by different authors in cattle and pig IVP embryos (Tominaga *et al.*, 2000; Li *et al.*, 2009).

As previous described a higher cryosensitivity was observed during the initial stages of development, from oocytes to late embryos, with the acquisition of a superior cryotolerance in an intermediate stage of development. In fact the large size of oocytes negatively influences the penetration of the cryoprotectant being these gametes particularly difficult to cryopreserved successfully (Vajta and Nagy, 2006; Pereira and Marques, 2008). Likewise Lin *et al.* (2011) found differences in the cryotolerance of rabbit *in vitro* produced embryos, vitrified by OPS, depending on its developmental stage, with better results in morulae/blastocysts than in the initial stage. These authors defined the 8-cell stage as the critical point for acquiring cryotolerance and the same was stated by Garcia-Garcia *et al.* (2006) in sheep embryos. Therefore it is not surprising that in the last years of research, cryopreservation techniques have focused mainly in embryos at morulae and blastocysts stages and less in oocytes. In addition embryos that develop early have better survival rates after cryopreservation and usually these are male embryos (Nedambale *et al.*, 2004).

2.5 Evaluation of cryopreserved embryos

Collection, production or storage of embryos aim to give practitioners the possibility of using them as an important resource for applying several reproductive techniques as indicated before. The effective use however must guarantee that these embryos have enough quality enabling the reliability of its use. The need of predicting embryo capacity in producing an offspring is essential in this context, reason why embryo morphology, post-thawing viability and blastocoele re-expansion have been widely used to determine its feasibility (Dattena *et al.*, 2000, 2004; Rizos *et al.*, 2004; Pereira *et al.*, 2007). To validate the application of all these techniques several methods can be used to predict success of IVP embryo survival. The ultimate method is, of course, the *in vivo* transfer of IVP embryos as the final goal of all the practical applications. Alternatives that involve less cost, lower concerns in animal welfare issues or laboratorial use include embryo thawing and culture with or without the use of complementary methods to estimate embryo viability. In this latter techniques electron microscopic evaluation or staining methods, can be included as we will mention later.

Embryo evaluation can use invasive and non-invasive techniques (Bączkowski *et al.*, 2004). The last group, in assessing embryos without damage, has been widely used based on morphological changes that occur as the result of the sequential cleavage and this classification can be important in preimplantation embryos and especially in embryos that are being transferred (Van Soom *et al.*, 2001). Ushijima *et al.*

INTRODUCTION

(2009), proposed an 11 stage classification for bovine embryos based on the number of cleavages. Embryo stage and quality that relate to morphology are usually based on the descriptions published by the IETS (Stringfellow and Seidel Jr, 1998). This ranking classifies embryos in grade 1 (excellent and good), grade 2 (fair) and 3 (poor) and become the standard reference worldwide. In cryopreserved embryos stereomicroscopic evaluation of morphology after thawing has been used for transfer selection (Abe *et al.*, 2002) to avoid transferring non-viable embryos and Leoni *et al.* (2008) stated that re-expansion of blastocoelic cavity within 8 h after vitrification/warming can be considered a reliable marker of its quality and developmental potential both *in vitro* and *in vivo*. However some authors argue that morphological evaluation of thawed embryos is not accurate and use of direct transfer of embryos can result in an improvement of 7-8% in offspring born (Cognié *et al.*, 2003). Also Green *et al.* (2009) found that direct transfer improves viability of transferred vitrified sheep embryos maybe because increasing the period from warming to transference has a detrimental effect on subsequent embryo viability. This fact could suggest that embryo evaluation after embryo warming is dispensable as predictive of success. In fact besides embryo morphological evaluation used in practice it is necessary to find other associated evaluation methods that can provide more accurate information. For example, Hernandez-Ledezma *et al.* (1993) showed that good quality hatched blastocysts produced more trophoblast protein thus being an indicator of embryo quality before transfer.

Selection of embryos by morphological criteria that is usually used has, as expected, some individual variations because can be a subjective evaluation. Indeed, studies in *in vivo* produced ovine morulae and blastocysts have shown that certain abnormalities remain undetected by stereomicroscopy (Cocero *et al.*, 2002).

Invasive methods of embryo evaluation are used mainly for research proposes, validating described techniques because they allow greater accuracy (Van Soom *et al.*, 2001). Staining methods as propidium iodide and TUNEL assay (Knijn *et al.*, 2003; Fabian *et al.*, 2005) are used for evaluating the number of viable cells. Hosseini *et al.* (2007) established an easy approach for *in vivo* derived and IVP embryos by evaluating the competence of cell membrane that is maintained in viable cells, and can differentiate them from those necrotic or apoptotic even without other morphological signs of cell dead (Betts and King, 2001).

Ultrastructural evaluation of embryos can predict the damage caused in cell structures by cryopreservation and the consequences in their functions (Smith and Silva, 2004), although this is an expensive and invasive technique but with important outputs in research because of detailed information.

Vajta *et al.* (1997) have observed, in IVP bovine embryos, relevant ultrastructural changes immediately after thawing, progressively restored over a 24-hr period. Also using ultrastructure observation Crosier *et al.* (2000, 2001) described morphometric techniques for the evaluation of bovine morulae and embryos.

Research on sheep embryo ultrastructure is limited but there are now enough information allowing discussion in this species. Recently Bettencourt *et al.* (2014) described the ultrastructure of sheep embryos produced *in vivo* and previously there were also some insights about the effects of cryopreservation in this source of embryos (Ferrer *et al.*, 1995; Cocero *et al.*, 2002; Rizos *et al.*, 2002a; Bettencourt *et al.*, 2009), although details about IVP sheep embryos fresh or cryopreserved is still needed. IVP sheep embryos lack desmosomal junctions, have a reduction in the microvilli, an increase of debris in the perivitelline space and high amount of lipid drops as referred before (Rizos *et al.*, 2002a). Ultrastructure of cryopreserved sheep embryos show signs of injury represented by the already referred differences in mitochondria, with presence of immature ones and in lower number in cryopreserved embryos (Cocero *et al.*, 2002; Bettencourt *et al.*, 2009). Mitochondria are important for embryo development and metabolism (Dorland *et al.*, 1994; Crosier *et al.*, 2000, 2001; Abe *et al.*, 2002) and thus can be used as a predictor of the potential development of embryos (Bettencourt *et al.*, 2009, 2014) but in cryopreserved sheep embryos their function is reduced or absent (Dalcin *et al.*, 2013). Cryopreserved embryos also show cytoskeleton disturbance with actin microfilament disorganization (Cocero *et al.*, 2002; Dalcin *et al.*, 2013) that can be caused by cryoprotectants (Dobrinsky, 1996).

Having in mind that evaluation of IVP embryos is important as prognostic for the success rate of embryo transfer it is necessary to search for more accurate and practical methods that ensure proper confidence, especially when dealing with stored cryopreserved embryos.

3 Published preliminary results

***In vitro* production of ovine embryos using fresh semen can improve blastocyst quality in Portuguese Merino breed**

Presented as oral communication in the IV Congress of Sociedade Portuguesa de Ciências Veterinárias, Vale de Santarém, Portugal, 2008.

Romão R, Marques CC, Baptista MC, Vasques MI, Barbas JP, Horta AEM, Bettencourt E, Pereira RM. A utilização de sémen fresco na fertilização *in vitro* de embriões ovinos melhora a qualidade dos blastocistos na raça portuguesa Merino. Livro de Resumos do IV Congresso da Sociedade Portuguesa de Ciências Veterinárias. INRB/INIA, Vale de Santarém, Portugal: 28, 2008.

Cryopreservation of ovine *in vitro* produced embryos using centrifugation and cytochalasin D

Presented as poster in the European Society for Domestic Animal Reproduction (ESDAR) Conference, Bologna, Italy, 12-14 September 2013.

Romão R, Marques C, Baptista M, Barbas J, Horta A, Carolino N, Bettencourt E, Pereira R. Cryopreservation of ovine *in vitro* produced embryos using centrifugation and cytochalasin D. *Reprod Dom Anim* 2013;48(Suppl.1):104.

3.1 *In vitro* production of ovine embryos using fresh semen can improve blastocyst quality in Portuguese Merino breed

R Romão¹, C Marques², MI Vasques², MC Baptista², J Barbas², A Horta², E Bettencourt¹, R Pereira²

¹Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Évora, Portugal;

²Instituto Nacional de Investigação Agrária e Veterinária, Vale de Santarém, Portugal

Embryo production in sheep is a difficult task demanding experience and expensive facilities, particularly when dealing with *in vivo* embryo production. Easy ways to obtain ovine embryos consist of collecting oocytes at slaughterhouses or systematically pick them up from live animals, allowing a large scale and cheaper *in vitro* embryo production (IVP) for small ruminants. Those are important sources of embryos, oocytes and zygotes for commercial, laboratorial and research proposes, making easier the availability of resources for emerging techniques like cloning or transgenesis. For IVP, several oocyte maturation protocols have been developed using fertilization (IVF) either with fresh or frozen-thawed semen. In Portugal, IVP has been done through IVF using cryopreserved semen because it is easily available for routine use. However, the use of fresh semen could improve embryo production and cryopreservation results. The aim of this work was to compare the efficiency of *in vitro* embryo production in ovine using different oocyte maturation protocols and fresh or frozen semen for IVF. Abattoir-derived oocytes (n=1768) were matured in TCM199, 10µM cysteamine, 10ng mL⁻¹ EGF, 10µg mL⁻¹ E2 and gentamicin (mat A, n=692) or plus 10µg mL⁻¹ FSH and 0.3 mM sodium piruvate (mat B, n=707) at 39°C and 5% CO₂ for 22h. Prior to fertilization, either fresh (FS) or frozen/thawed (TS) semen from Merino rams (n=3) was washed or submitted to swim-up respectively. Presumptive zygotes (18h p.i.) were cultured in synthetic oviductal fluid (SOF) enriched with aminoacids and 6 mg mL⁻¹ BSA at 38.5°C, under 5% O₂, 5% CO₂ and 90% N₂ in an humidified atmosphere until the stage of 2-4-8 cell embryos. After assessing cleavage, embryo development proceeded until the blastocyst stage in SOF+BSA and 10% FCS. Quality was evaluated on D6-7 by scoring embryos as good, fair and bad based on IETS guidelines. Data from embryo production rates were analysed using ANOVA. Mann-Whitney U test was used for embryo quality evaluation. Different maturation protocols did not interfere (P>0.05) either on maturation or on embryo quality or production rates. Embryo quality was higher (P=0.004) when fertilization was accomplished with fresh semen (good: FS=40.1±8.0% vs TS=32.9±5.6%; fair: FS=20.1±4.7% vs TS=35.7±5.8%; bad: FS=39.8±9.8% vs TS=31.4±7.6%). Preliminary results shows that ram fresh semen can be easily used for *in vitro* fertilization and improves the quality of produced embryos.


3.2 Cryopreservation of ovine *in vitro* produced embryos using centrifugation and cytochalasin D

R Romão¹, C Marques², M Baptista², J Barbas², A Horta², N Carolino², E Bettencourt¹, R Pereira²

¹Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Évora, Portugal;


²Instituto Nacional de Investigação Agrária e Veterinária, Vale de Santarém, Portugal

The cryosurvival of ovine *in vitro* produced embryos are still low, thus preventing its routine transfer. Attempts have been made to override this problem by decreasing embryos lipid content or protecting their structure with cytoskeletal stabilizers. In this study we used embryo mechanical delipidation through centrifugation in the presence or absence of cytochalasin D testing its effect on embryo quality and cryosurvival. Mature ovine oocytes (n = 1146) were inseminated using fresh semen of a Merino ram. After assessing cleavage, embryo development proceeded until the blastocyst stage. Prior to vitrification, embryos were randomly distributed to the following groups: control (n = 20), without treatment; centrifugation (n = 18), blastocysts were centrifuged at 15 000 X g; cytochalasin D (n = 20), embryos were treated with 5 µg/ml cytochalasin D; centrifugation + cytochalasin D (n = 17), embryos were treated with both centrifugation and cytochalasin D. Embryos integrity and re-expansion were assessed post-warming and after 3 h of culture. Post-warming integrity rate was lowest (p=0.04) in embryos of centrifugation group. No differences were identified among groups for re-expansion rates. A possible role of cytochalasin D in protecting mechanical damage of centrifuged embryos during cryopreservation was identified. However this stabilizer alone did not improve the quality of warmed embryos when compared to control.



Cryopreservation of ovine *in vitro* produced embryos using centrifugation and cytochalasin D

R. Romão ¹, C. Marques ², M. Baptista ², J. Barbas ², A. Horta ², N. Carolino ², E. Bettencourt ¹ and R. Pereira ²



¹ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Escola de Ciências e Tecnologia, Universidade de Évora Núcleo da Mitra, Ap. 94, 7002- Évora, Portugal
rromao@uevora.pt; emvb@uevora.pt

²Instituto Nacional de Investigação Agrária e Veterinária, I.P., Unidade Estratégica de Investigação e Serviços de Biotecnologia e Recursos Genéticos, Polo de Investigação da Quinta da Fonte Boa Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal
rosalinda@uevora.pt; baptista.sao@gmail.com; jbarbas@gmail.com; horta.antonio@mail.telepac.pt; carolinonuno@hotmail.com; rosalnp@gmail.com

Introduction


The cryosurvival of ovine *in vitro* produced embryos are still low, thus preventing its routine transfer. Attempts have been made to override this problem by decreasing embryos lipid content or protecting their structure with cytoskeletal stabilizers. In this study we used embryo mechanical delipidation through centrifugation in the presence or absence of cytochalasin D testing its effect on embryo quality and cryosurvival.

Results

Post-warming integrity rate was lowest ($p=0.04$) in embryos of centrifugation group (figure 1). No differences were identified among groups for re-expansion rates after thawing and 3 hours of culture.

Treatment Group	Blastocyst Integrity (%)	Significance
centcytoD	~88	a
cent	~55	b
cytoD	~85	a
control	~65	a

Figure 1: Blastocyst integrity after warming of the four treatment groups: centcyto= centrifugation + cytochalasin D; cent=centrifugation; cytoD=cytochalasin D (a≠b, $p<0,01$)



Conclusion

A possible role of cytochalasin D in protecting mechanical damage of centrifuged embryos during cryopreservation was identified. However this stabilizer alone did not improve the quality of warmed embryos when compared to control.

Materials and methods

Mature ovine oocytes ($n=1146$) were inseminated using fresh semen of a Merino ram. After assessing cleavage, embryo development proceeded until the blastocyst stage. Prior to vitrification by OPS, embryos were randomly distributed to the following groups: control ($n= 20$), without treatment; centrifugation ($n=18$), blastocysts were centrifuged at 15000g; cytochalasin D ($n=20$), embryos were treated with 5 $\mu\text{g mL}^{-1}$ cytochalasin D; centrifugation+cytochalasin D ($n=17$), embryos were treated with both centrifugation and cytochalasin D. Embryos integrity and re-expansion were assessed post-warming and after 3 hours of culture. Data were analysed by logistic regression (PROCLOGISTIC, SAS) with a model including the effect of session, embryo quality and stage at cryopreservation. Additionally we calculated the odds ratio between different treatments and its contrasts.

This work was supported by FCT through the Portuguese Foundation for Science and 330 Technology (PPTDC/CVT/98607/2008) and FCT Grant (SFRH/BD/37853/2007).

European Society for Domestic Animal Reproduction (ESDAR) CONFERENCE, 12-14 September 2013, Bologna, Italy

Figure 1: sample of poster (100x70 cm) presented in the ESDAR conference 2013, 12-14 September Bologna, Italy.

4 Published papers

Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen

Romão R, Marques CC, Baptista MC, Vasques MI, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Plancha C, Rodrigues P, Pereira RM. Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen. Small Ruminant Research 2013;110:36-41.

4.1 Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen

R. Romão^a, C.C. Marques^b, M.C. Baptista^b, M.I. Vasques^b, J.P. Barbas^b, A.E.M. Horta^b, N. Carolino^{b,d},
E. Bettencourt^a, C. Plancha^c, P. Rodrigues^c, R.M. Pereira^{b,d}

^a Escola de Ciências e Tecnologia, Universidade de Évora, Polo da Mitra, Apartado 94, 7002-554 Évora, Portugal

^b Unidade de Recursos Genéticos, Reprodução e Melhoramento Animal, INRB IP, L INIA-Santarém, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal

^c Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina da Universidade de Lisboa, Av. Professor Egas Moniz, 1649-028 Lisboa, Portugal

^d Escola Universitária Vasco da Gama, Mosteiro de S. Jorge de Milréu, Estrada da Conraria, 3040-714 Castelo Viegas, Coimbra, Portugal

Abstract

Successful production of high quality blastocysts depends on the use of a culture system that ensures the acquisition of developmental competence by the maturing oocyte followed by an efficient *in vitro* fertilization. In the present work the effect of FSH and pyruvate in an EGF containing medium for ovine oocyte maturation prior to insemination with fresh (F) or frozen–thawed (FT) semen on embryo developmental competence and cryosurvival was determined. Sheep oocytes were matured in two culture media (M1 and M2, respectively; M1 = CM + EGF, n = 836 and M2 = CM + EGF + pyruvate + FSH, n = 850) for 22 h and then fertilized using FT or F spermatozoa (M1×FT = 371, M2×FT = 359, M1×F = 353 and M2×F = 372, 9 replicates) from Merino rams (n = 3). After embryo culture and evaluation, good quality blastocysts (grade 1) were vitrified in OPS. Post-thawed embryo integrity, re-expansion and number of total and viable cells were assessed. Oocyte maturation rates presented no differences ($p > 0.05$) between treatments (M1 = 87.0 ± 4.1 and M2 = $86.7 \pm 3.9\%$) as well as embryo developmental rates either for maturation media or semen status. However, fresh semen improved blastocyst quality (grade 1 embryos F = $52.5 \pm 4.8\%$ and FT = $39.0 \pm 4.4\%$, $p = 0.01$). Grade 1 blastocysts presented similar post-thawed integrity and re-expansion rates. After 3 h of culture, expansion rates were higher ($p = 0.05$) for M2×F warmed embryos ($80.0 \pm 8.3\%$) than for M1×F ($54.3 \pm 10.4\%$). Results seem to confirm the existence of a synergistic effect between FSH, EGF and pyruvate upon cytoplasmic maturation of ovine oocytes. Moreover, *in vitro* fertilization by fresh semen clearly improves ovine embryo developmental competence by enhancing morphological blastocyst quality. The beneficial effect of M2 on cryosurvival was only observed in embryos derived from fresh semen. Therefore these combined strategies enhance embryo cryosurvival.

Keywords: ovine; fresh semen; frozen semen; oocyte maturation; *in vitro* embryos; cryopreservation

4.1.1 Introduction

Embryo production in small ruminants is a difficult task demanding experience and expensive facilities. The relative inefficiency of *in vivo* embryo production limits its use in sheep (Thibier and Guérin, 2000). In spite of the efforts towards the improvement of *in vitro* techniques by collecting oocytes in slaughterhouses or systematically pick them up from live animals, embryo production rates are still far from ideal, and transferable blastocysts are inferior in quality and cryotolerance compared to their *in vivo* counterparts (Papadopoulos *et al.*, 2002; Cognié *et al.*, 2003).

Several oocyte maturation protocols have been developed for *in vitro* fertilization (IVF) either with fresh or frozen-thawed semen (Dattena *et al.*, 2004; Pereira *et al.*, 2009). Regarding oocyte maturation, differences exist in the developmental competence of those matured *in vivo* compared to the *in vitro* ones (van de Leemput *et al.*, 1999). Furthermore oocytes that have been exposed to a gonadotropin stimulus either *in vivo* or during *in vitro* culture are more competent to support embryonic development following fertilization (Rizos *et al.*, 2002; Farin *et al.*, 2007). The presence of receptors for growth factors such as insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) in granulosa cells and oocytes suggest their involvement in the maturation process and developmental competence (Feng *et al.*, 1987; Wang *et al.*, 2009; Procházka *et al.*, 2011) at least in early stages (Kelly *et al.*, 2008). However, completion of meiosis in ovine oocytes and embryo development were significantly stimulated by the presence of FSH or EGF in the maturation medium, whereas IGF-1 was not effective (Guler *et al.*, 2000). Recently a synergistic effect of FSH and EGF on cytoplasmic maturation of porcine oocytes was reported by Uhm *et al.* (2010). Thus, it is possible that FSH and EGF work in concert to regulate oocyte maturation and optimize oocyte developmental competence *in vitro*. During oocyte maturation, FSH and EGF promote glucose metabolism in cumulus cells through the hexosamine biosynthetic pathway (HBP) leading to cumulus expansion by the synthesis of extracellular matrices (Buccione *et al.*, 1990). On the other hand, the oocyte has a poor capacity to use glucose which must be metabolized to pyruvate by the cumulus cells to supply the oocyte. Sugiura *et al.* (2005) suggest that FSH/EGF stimulation of HBP during *in vitro* maturation denies the oocyte-mediated promotion of glycolysis that was seen within the cumulus cells resulting in an energy imbalance in the oocyte. Pyruvate supplementation should minimize this fact.

Moreover, reports comparing IVP embryos competence and cryotolerance after IVF using fresh or cryopreserved sperm are sparse. Although cryopreserved ram semen is easily available for routine use,

PUBLISHED PAPERS

freezing and thawing disrupts the stability of ram sperm chromatin, most likely reducing the fertilization efficiency of cryopreserved semen *in vitro* and *in vivo* (Peris *et al.*, 2004). Consequently the use of fresh semen could improve embryo production and cryopreservation results. The purpose of this study was to determine the effect(s) of the inclusion of FSH and pyruvate in an EGF containing maturation medium as well as the use of fresh and frozen–thawed sperm for fertilization on ovine embryo developmental competence and cryosurvival.

4.1.2 Materials and methods

All chemicals used were purchased from Sigma Aldrich Chemical Co. (St. Louis, USA) unless specified otherwise.

4.1.2.1 Experimental design

In this experiment, 1686 oocytes collected from 859 ovaries were used to produce ovine embryos that were subjected to a 2x2 factorial experimental design with two maturation media (M1, maturation medium usually used in our Lab as in Pereira *et al.* (2009), 836 immature oocytes; or M2, M1 plus FSH and Na pyruvate, 850 immature oocytes) and two semen treatments (fresh-F or frozen–thawed-FT). The three Merino rams used and sessions or batch of ovaries were considered random effects. Each ram was tested three times running once individually and twice simultaneously with each other, performing a total of 9 replicates. Morphological parameters as well as the capacitation status of fresh and frozen–thawed ram semen prior to IVF were evaluated. Cleavage was assessed by microscopy 48 h after fertilization (IVF = day 0). On day 6 till day 8, embryos were evaluated for developmental and morphological status (grade 1, good and grade 3, poor). Grade 1 embryos (M1xFT, n = 19; M2xFT, n = 27; M1xF, n = 30; M2xF, n = 29) were vitrified. After warming (3 sessions), embryo viability was evaluated by its integrity and re-expansion as well as after 3 h of *in vitro* culture. The number of cells (total and viable) in thawed/cultured blastocysts (2–6 embryo per treatment in each session) was also assessed.

4.1.2.2 Oocyte *in vitro* maturation

Ovaries collected from crossbred Merino sheep at a local slaughterhouse were transported to the laboratory in Dulbecco's phosphate buffer saline (PBS, GibCo 14040-91) at 35°C. PBS was supplemented with 0.15% (w/v) of bovine serum albumin (BSA) and 0.05 mg mL⁻¹ of kanamycin. At the laboratory, the 2–6 mm follicles were aspirated to obtain the cumulus-oocyte complexes (COC).

Selected COC were divided and incubated in two different maturation media (M1 and M2) at 38.5°C and 5% CO₂ for 22 h. The composition of first medium (M1) included medium 199, 10µM cysteamine, 10 ng mL⁻¹ EGF, 10 µg mL⁻¹ estradiol and 10 µL mL⁻¹ gentamicin (Pereira *et al.*, 2009). The second medium (M2) was also supplemented with 0.3 mM sodium pyruvate and 10 µg mL⁻¹ FSH.

The apparent maturation was evaluated (mature oocytes scored morphologically/immature oocytes). Oocytes were considered mature if a homogenous cytoplasm and multilayered, expanded cumulus cells were present.

4.1.2.3 Semen collection

Semen collection was conducted at the experimental farm of INRB in compliance with the requirements of the European Union for farm animal welfare and the Portuguese authority guidelines for animal experimentation.

Semen was collected from three Portuguese Merino rams of proven fertility by an artificial vagina. Each ejaculate was immediately evaluated for volume, motility and concentration. Only semen with good quality (mass motility > 4; individual motility > 60%; concentration > 2.5×10⁹ spz mL⁻¹) was used either fresh or cryopreserved.

4.1.2.4 Semen cryopreservation

Ejaculates with good quality were diluted in a solution of 45.0 g L⁻¹ Tris, 24.4 g L⁻¹ citric acid (Merck 1.002.441.000), 5.6 g L⁻¹ glucose, 15% egg yolk (v/v), 6.6% glycerol (v/v, Merck 1.040.011.000) and antibiotics (Marques *et al.*, 2006). After dilution, semen was packed in 0.25 mL Cassou straws (300×10⁶ spz) and refrigerated (4°C) for 4 h prior to freezing in liquid nitrogen (LN₂) vapor during 25 min and then submersed and kept in a LN₂ container (Valente *et al.*, 2010).

4.1.2.5 Preparation and evaluation of fresh and thawed semen

After collection, fresh ejaculates were kept at room temperature (22°C) and light protected for up to 2 h, then washed in synthetic oviductal fluid (SOF) and centrifuged at 225×g for 5 min. This medium was SOF enriched with bovine serum albumin (4 mg mL⁻¹ BSA) plus glutamine (1.5 µg mL⁻¹) and kept at the same room temperature (adapted from Dattena *et al.*, 2000). Subsequently washed spermatozoa were diluted with 1 mL of fertilization medium consisting of SOF containing BME (20 µL mL⁻¹) and MEM amino acids (10 µL mL⁻¹), gentamicin (10 µL mL⁻¹) and 10% ovine superovulated oestrus serum.

PUBLISHED PAPERS

After thawing, sperm motility was immediately examined. Frozen–thawed semen was then incubated at 38.5°C and 5% CO₂ for 1h in capacitation medium (modified Brackett's medium containing 20% ovine superovulated oestrus serum, Pereira *et al.*, 2009). After centrifugation at 225×g for 5 min, the supernatant were rejected and the remaining pellet of spermatozoa evaluated prior to be used to fertilize the oocytes in fertilization medium.

4.1.2.5.1 Morphological parameters

Either post swim-up thawed or washed fresh sperm were evaluated for individual motility percentage of progressively motile spermatozoa), vigor (scale 0–5) and concentration.

4.1.2.5.2 Capacitation

The capacitation status of swimm-up thawed or washed fresh sperm were assessed by chlortetracycline (CTC) staining binding pattern in the spermatozoa membranes using the technique described by Pereira *et al.* (2009). Aliquots (5 µL) of motile spermatozoa were mixed with 5 µL of CTC solution (0.4 g L⁻¹ CTC–HCl, 0.9 g L⁻¹ cystein in 5 mL of 2.4 g L⁻¹ Tris and 7.6 g L⁻¹ NaCl solution), 1 µL of 12.5% glutaraldehyde solution and 1 µL of DABCO (Merck 8.03456). Slides were observed under fluorescence within 12 h and spermatozoa classified according to their acrosomal status as: uncapacitated acrosome intact; capacitated acrosome intact; and acrosome reacted cells.

4.1.2.6 *In vitro* fertilization and embryo culture

Procedures were as described by Pereira *et al.* (2009). Briefly, sheep matured COC were co-cultured with fresh or thawed spermatozoa (1×10⁶ spz mL⁻¹) in SOF containing BME and MEM amino acids, gentamicin and 10% ovine superovulated oestrus serum as described above.

Eighteen hours after insemination, presumptive zygotes were transferred into 25 µL droplets of SOF supplemented with BME and MEM amino acids and bovine serum albumin (6 mg mL⁻¹ BSA) until the stage of 2-8 cells. After assessing cleavage using a stereo microscope (Olympus SZ60), embryo development proceeded until the blastocyst stage in amino acids and BSA supplemented SOF plus 10% fetal calf serum (SOFserum). Embryo culture was performed at 38.5°C in a humidified atmosphere with 5% O₂, 5% CO₂ and 90% N₂.

Cleavage rate was calculated as the number of cleaved embryos per number of inseminated oocytes. Day 6 (D6) and D8 embryo developmental rates were calculated as the number of D6 or D8 blastocysts per number of cleaved embryos, respectively.

Embryo quality (D6–D8) was classified on the basis of conventional morphological criteria and according to their stage of development following the guidelines of the International Embryo Transfer Society (grade 1: good, no blemishes or only trivial imperfections; grade 2: fair, with some extruded or degenerated cells and non-uniform, darker appearance; and grade 3: bad, poor quality, lacking cohesion or with many extruded or degenerated cells).

4.1.2.7 Blastocyst vitrification and warming

A proportion of grade 1 blastocysts was vitrified using the open-pulled straw (OPS) method described by Vajta *et al.* (1998). Blastocysts were first equilibrated in holding medium (HM, M199 + 20% FCS) for 5 min, then in diluted (HM with 10% ethylene glycol-EG and 10% dimethyl sulfoxide- DMSO, 1 min) and concentrated (HM with 20% EG and 20% DMSO) vitrification media. Embryos were quickly placed into the superfine OPS and plunged directly into NL₂ (30 s). Warming was performed by placing the end of the straw directly into the holding medium. After 2 min, blastocysts were transferred into HM for another 2 min; and then to SOFserum and cultured for 3 h. Samples of these embryos (2–6 embryo per treatment in each post-thawed session) were fixed and stained for cell number and viability evaluation.

Embryos integrity and re-expansion (Pereira *et al.*, 2007) were assessed 20 min post-thawing and after 3 h of *in vitro* culture (Morató *et al.*, 2011). Embryo integrity rate was calculated as the number of intact embryos (blastocysts with an intact zona pellucida and without several extruded blastomeres lacking cohesion) per number of frozen–thawed embryos. Embryo re-expansion rate was calculated as the number of reexpanded embryos (blastocysts that recovered their original shape with re-expanded blastocoels) per number of frozen–thawed embryos.

4.1.2.8 Evaluation of embryo cell viability and total number

After culture, post-thawed grade 1 blastocysts were washed in PBS, transferred into pronase containing medium for 1.5 min and then to Tyrodes medium for another 1.5 min. Afterwards the embryos without the zona pelucida were washed again in PBS and incubated in SOFserum for 1 h prior to fixation in a 4% paraformaldehyde solution and staining in a solution of 50 µg mL⁻¹ of Hoechst 33342 and 5 µg mL⁻¹ of propidium iodide (PI) (adapted from Hosseini *et al.*, 2007).

Finally embryos were individually placed into 2 µL of Mowiol (Calbiochem 475904) medium on glass slides and covered with a coverslip. Slides were refrigerated and kept in dark until being observed in a widefield fluorescence microscope (Leica DM5000B) using red and ultraviolet excitation. Images were saved

using the software Image J (National Institutes of Health, USA) and total cell number (Hoechst 33342 stain) and dead cells (PI stain) counted.

4.1.2.9 Statistical analysis

Procedure MIXED of Statistical Analysis Systems Institute (SAS Inst., Inc., Cary, NC, USA) was used to analyze data with a model including: embryo production (cleavage, D6 and D8 embryos rates) as well as the number of cells in each post-thawed embryo and their viability. Mixed linear model included *in vitro* maturation media and semen status as fixed effects and combination of ram*replicate as random effect. In addition the means for each treatment were calculated as well as the differences between the means and the respective t-test. Proc MIXED of SAS was also used to analyze data from apparent maturation and semen evaluation with a model including *in vitro* maturation media or semen status as fixed effects, respectively and in the latter case the combination ram*replicate as random effect.

Data from embryo quality (good, fair and bad) and frozen–thawed embryos survival were analyzed using GLIMMIX procedure from SAS with a model that included the effect of *in vitro* maturation media and semen status as fixed effects and ram as random effect.

Results were considered statistically different when $p \leq 0.05$.

4.1.3 Results

The apparent maturation rates presented no differences ($p > 0.05$) between treatments (M1 = 87.0 ± 4.1 and M2 = $86.7 \pm 3.9\%$). Equally, no differences were found among rams ($n = 3$) semen parameters prior to IVF. However, as expected, initial ($p < 0.0001$) and pre-IVF ($p = 0.05$) motility and vigor ($p = 0.04$) in fresh semen were superior to those of frozen-thawed semen (Table 1). The capacitation status of swimmable spermatozoa presented no differences ($p > 0.05$) between fresh and frozen–thawed semen.

Table 1: Evaluation of morphological parameters and capacitation status (chlortetracycline staining-CTC) of fresh (F) and frozen thawed (FT) ovine semen (least squares means \pm standard error).

Semen status	N	Morphological parameters			CTC		
		Initial motility (%)	Pre-IVF motility (%)	Vigor (0-5)	NCap (%)	Cap (%)	AR (%)
F	9	75.2 ± 1.8^a	77.4 ± 3.5^a	4.7 ± 0.1^a	39.2 ± 3.4	41.9 ± 2.9	19.8 ± 2.8
FT	9	45.5 ± 1.8^b	66.3 ± 3.5^b	4.4 ± 0.1^b	28.8 ± 3.4	47.0 ± 2.9	24.1 ± 2.8

Data within the same columns with different superscripts letters are statistically different ($p \leq 0.05$); NCap: uncapacitated acrosome intact cells; Cap: capacitated acrosome intact cells; AR: acrosome reacted cells; IVF: in vitro fertilization.

Similar embryo production rates were obtained in all groups (Table 2), without significant effects of maturation media and semen status or interaction between them.

Table 2: Effect of different maturation media (M1 and M2) and oocyte fertilization by fresh (F) or frozen–thawed (FT) spermatozoa on embryo production rates (least squares means \pm standard error and p-values).

Treatment	N	Cleavage (%)	D6 embryo (%)	D8 embryo (%)
M1 \times FT	371	48.0 \pm 5.4	46.9 \pm 3.8	38.5 \pm 3.2
M2 \times FT	359	45.1 \pm 3.5	44.5 \pm 3.0	42.8 \pm 3.3
M1 \times F	353	44.2 \pm 4.7	42.6 \pm 4.1	37.3 \pm 5.3
M2 \times F	372	41.9 \pm 5.1	43.0 \pm 3.7	37.4 \pm 5.5
Total	1455	44.8 \pm 2.3	44.24 \pm 1.8	39.0 \pm 2.2
Effects	M	p=0.358	p=0.382	p=0.414
	S	p=0.495	p=0.762	p=0.594
	M \times S	p=0.939	p=0.666	p=0.614

M: maturation media; M1: maturation medium containing EGF; M2: maturation medium containing EGF, pyruvate and FSH; N: inseminated oocytes; S: semen status.

Grade 1 embryos rate using F semen was higher ($p=0.01$) than with FT semen (54.6 \pm 7.7% and 39.5 \pm 7.3%, respectively). Interestingly, there was no significant effect of the maturation media or interaction between maturation media and semen status on this parameter (Figure 2). However this interaction was

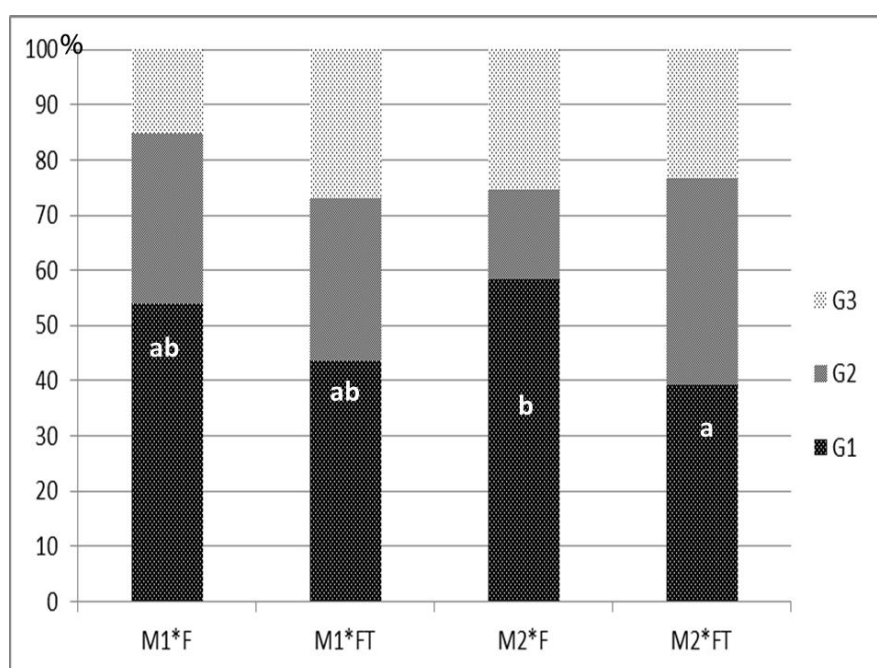


Figure 2: Percentual distribution of embryo (n=279) morphological quality [grade 1 embryos (G1); grade 2 embryos (G2); grade 3 embryos (G3)] in the four groups according to maturation method (M1: maturation medium containing EGF and M2: medium containing EGF, pyruvate and FSH) and type of semen used: fresh (F) or frozen–thawed (FT) (a \neq b, P=0.007).

significant ($p=0.03$) for grade 2 embryos being the rate of these blastocysts lower ($p=0.007$) in M2×F (19.3±5.6%) group than in M2×FT (40.0±5.5%). The maturation media and semen status did not influence ($p>0.05$) on grade 3 blastocyst rate.

One hundred and five good quality embryos were vitrified and warmed to evaluate post-viability (Table 3). After warming, similar results were obtained in embryo integrity and re-expansion rates. However, after 3 h of *in vitro* culture, the expansion rate was higher in M2×F than in M1×F. No differences were found in embryo total cells number or in their viability among treatments.

Table 3: Effect of different maturation media (M1 and M2) and oocyte fertilization by fresh (F) or frozen–thawed (FT) spermatozoa on post-thawed embryo (n=105) viability evaluated by intact and re-expanded blastocyst rates and embryo cell counts (least squares means ± standard error).

Treatments	Post-thawed			After culture	
	n	Intact embryos (%)	Re-expanded embryos (%)	Re-expanded embryos (%)	Total cells (viability)
M1 FT	30	80.7±8.3	77.4±6.9	74.7±8.9 ^{ab}	56.6± 8.2 (33.5%)
M2 FT	19	69.4±11.9	65.3±15.3	60.0±12.0 ^{ab}	59.4± 11.5 (20.3%)
M1 F	29	76.2±9.3	71.8±15.5	54.3±10.4 ^b	65.0± 8.6 (36.6%)
M2 F	27	73.2±15.5	69.0±10.1	80.0±8.3 ^a	48.9± 12.8 (39.2%)

Data within the same columns with different superscripts letters are statistically different ($p\leq 0.05$); M1: maturation medium containing EGF; M2: maturation medium containing EGF, pyruvate and FSH; N: inseminated oocytes.

4.1.4 Discussion

Although *in vitro* production and cryopreservation of ovine embryos can be an useful technique in reproductive biotechnology, the technical feasibility and quality of the produced embryos are lower than for other species (Thibier and Guérin, 2000; Pereira and Marques, 2008). Improvement of practical results could trigger a more extensive application for this technique. Our goal was to compare the use of two different maturation media and type of semen for IVF in order to optimize results. Data presented here demonstrated that fertilization of matured ovine oocytes with F semen improves the morphological quality of the produced embryos. Moreover an enhanced embryo cryosurvival is achieved when a maturation medium is supplemented with FSH, EGF and pyruvate, allied to the F semen used for IVF.

As referred, the individual effect of FSH/EGF or pyruvate on the cytoplasmatic maturation of the oocyte has been thoroughly investigated. Accordingly, FSH or EGF is largely used in many *in vitro* maturation protocols because it has been shown to improve cumulus expansion, oocyte cytoplasmatic maturation, fertilization and early embryonic development (Paria and Dey, 1990; Wang *et al.*, 2009). Recently a synergistic effect of FSH and EGF on cytoplasmic maturation of porcine oocytes was reported by

Uhm *et al.* (2010). Moreover we hypothesized that pyruvate could minimize the energy imbalance in the oocyte referred by Sugiura *et al.* (2005), due to the stimulation of HBP by FSH/EGF during *in vitro* maturation. Therefore by adding these components to the maturation media we tried to improve *in vitro* maturation of ovine oocytes through a synergistic effect of FSH, EGF and pyruvate. Although no differences were identified in maturation, cleavage and D6-D8 rates, FSH, EGF and pyruvate presence improved the oocyte developmental competence. In fact, after 3 h of culture, expansion rate of vitrified-warmed grade 1 quality embryos in M2×F group was higher ($p=0.05$) than in M1 × F group. Thus, it seems that FSH, EGF and pyruvate contribute to regulate ovine oocyte maturation and optimize their developmental competence *in vitro*, namely when using F semen for IVF.

Cryopreservation severely damages ram spermatozoa. Therefore as expected and previously documented (Bailey *et al.*, 2000; Marques *et al.*, 2006), the classical andrological sperm parameters were reduced in quality following cryopreservation. The observation that cryopreserved ram spermatozoa have more susceptible and heterogeneous chromatin than fresh, suggests that the poorer fertilizing efficiency of frozen ram semen might be at least partly due to abnormal sperm DNA structure, despite having a normal appearance soon after thawing (Peris *et al.*, 2004). Interestingly similar cleavage, D6 and D8 embryo rates were obtained independently of the semen status, F or post-thawed, in the present study. In accordance Lehloenya *et al.* (2010) also reported no significant differences between F and FT semen regarding the embryonic developmental stages although presenting a blastocyst rate ranging between $0.4\pm 0.4\%$ and $2.6\pm 1.0\%$, respectively.

Despite some previous results that had found no differences in the cleavage rates between oocytes inseminated with F or FT semen (Pugh *et al.*, 1991; Lehloenya *et al.*, 2010), present results demonstrate that the use F sperm for *in vitro* fertilization can improve ovine embryo developmental competence by enhancing morphological blastocyst quality. The inferior viability of *in vitro* produced embryos is attributed to cellular (Pereira *et al.*, 2007; Rizos *et al.*, 2002), metabolic (Khurana and Niemann, 2000; Farin *et al.*, 2001) and biochemical changes (Massip *et al.*, 1995). *In vitro* ovine blastocysts exhibited less microvilli and a less extensive network of intercellular junctions, specifically an apparent lack of desmosomal junctions, a higher incidence of cellular debris and a higher number of lipid droplet than their *in vivo* counterparts (Farin *et al.*, 2001; Rizos *et al.*, 2002). These differences could certainly contribute to the observed lower cryotolerance of *in vitro* produced embryos.

PUBLISHED PAPERS

Embryo morphology and post-thawing viability have been widely used to determine embryo viability (Dattena *et al.*, 2000, 2004; Pereira *et al.*, 2007). In addition to the above mentioned beneficial effects of FSH, EGF and pyruvate on *in vitro* maturation, FSH promotes the synthesis of hyaluronic acid (HA) in expanding cumulus cells during maturation. Moreover, HA synthesis during cumulus mucification contributes to the penetration and fertilization of oocytes, most likely facilitating the process of capacitation and acrosome reaction and subsequent developmental competence (Gutnisky *et al.*, 2007). This could explain that herein besides the improvement of blastocyst quality when using F semen for IVF, an interaction between semen status and maturation media on embryo quality and cryosurvival was identified. Whereas M2×F group presented less grade 2 embryos than M2×FT group, the expansion rate of vitrified-warmed grade 1 embryos from M2×F group was higher than in M1×F group. When using FT semen, unexpectedly no differences were identified in embryo post-thawed expansion rates. Thus the association of a maturation medium containing FSH, EGF and pyruvate with F semen for IVF should be used to enhance the viability of ovine *in vitro* produced embryos.

4.1.5 Conclusion

In conclusion presented results seem to confirm the existence of a synergistic effect between FSH, EGF and pyruvate on cytoplasmic maturation of ovine oocytes. In addition, *in vitro* fertilization by fresh semen clearly improves ovine embryo developmental competence by enhancing morphological blastocyst quality. The beneficial effect of M2 on cryosurvival was only observed in embryos derived from fresh semen. Therefore these combined strategies enhance embryo cryosurvival.

4.1.6 References

- Bailey, J.L., Bilodeau, J.F., Cormier, N., 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J. Androl.* 21, 1–7.
- Buccione, R., Vanderhyden, B.C., Caron, P.J., Eppig, J.J., 1990. FSH-induced expansion of the mouse cumulus oophorus *in vitro* is dependent upon a specific factor(s) secreted by the oocyte. *Dev. Biol.* 138, 16–25.
- Cognié, Y., Baril, G., Poulin, N., Mermillod, P., 2003. Current status of embryo technologies in sheep and goat. *Theriogenology* 59, 171–188.
- Dattena, M., Accardo, C., Pilchi, S., Isachenko, V., Mara, L., Chessa, B., Cappai, P., 2004. Comparison of different vitrification protocols on viability after transfer of ovine blastocysts *in vitro* produced and *in vivo* derived. *Theriogenology* 62, 481–493.
- Dattena, M., Ptak, G., Loi, P., Cappai, P., 2000. Survival and viability of vitrified *in vitro* and *in vivo* produced ovine blastocysts. *Theriogenology* 53, 1511–1519.
- Farin, C.E., Rodriguez, K.F., Alexander, J.E., Hockney, J.E., Herrick, J.R., Kennedy-Stoskopf, S., 2007. The role of transcription in EGF- and FSH-mediated oocyte maturation *in vitro*. *Anim. Reprod. Sci.* 98, 97–112.

- Farin, P.W., Crosier, A.E., Farin, C.E., 2001. Influence of *in vitro* systems on embryo survival and fetal development in cattle. *Theriogenology* 55, 151–170.
- Feng, P., Knecht, M., Catt, K.J., 1987. Hormonal control of epidermal growth factor receptors by gonadotropins during granulosa cell differentiation. *Endocrinology* 120, 1121–1126.
- Guler, A., Poulin, N., Mermillod, P., Terqui, M., Cognié, Y., 2000. Effect of growth factors, EGF and IGF-1, and estradiol on *in vitro* maturation of sheep oocytes. *Theriogenology* 54, 209–218.
- Gutnisky, C., Dalvit, G.C., Pintos, L.N., Thompson, J.G., Beconi, M.T., Cetica, P.D., 2007. Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte *in vitro* maturation, fertilisation and embryo development. *Reprod. Fertil. Dev.* 19, 488–497.
- Hosseini, S.M., Hajian, M., Asgari, V., Forozanfar, M., Abedi, P., Nasr- Esfahani, M.H., 2007. Novel approach of differential staining to detect necrotic cells in preimplantation embryos. *Iran. J. Fertil. Steril.* 1, 103–106.
- Kelly, J.M., Kleemann, D.O., Maxwell, W.M.C., Walker, S.K., 2008. Effects of insulin-like growth factor-I, epidermal growth factor and cysteamine on the *in vitro* maturation and development of oocytes collected from 6- to 8-week-old Merino lambs. *Reprod. Fertil. Dev.* 20, 570–578.
- Khurana, N.K., Niemann, H., 2000. Effects of cryopreservation on glucose metabolism and survival of bovine morulae and blastocyst derived *in vitro* or *in vivo*. *Theriogenology* 54, 313–326.
- Lehloenya, K.C., Mahoete, N., Greyling, J.P.C., Nedambale, T.L., 2010. Effect of breed and frozen–thawed ram semen on *in vitro* fertilization and ovine embryonic development. *Reprod. Fertil. Dev.* 23, 170.
- Massip, A., Mermillod, P., Dinnyes, A., 1995. Morphology and biochemistry of *in vitro* produced bovine embryos: implications for their cryopreservation. *Hum. Reprod.* 10, 3004–3011.
- Marques, C.C., Barbas, J.P., Baptista, M.C., Cannas-Serra, C., Vasques, M.I., Pereira, R.M., Cavaco-Gonçalves, S., Horta, A.E.M., 2006. Reproduction in the ovine Saloia breed: seasonal and individual factors affecting fresh and frozen semen performance, *in vivo* and *in vitro* fertility. In: Ramalho Ribeiro, J.M.C., Horta, A.E.M., Mosconi, C., Rosati, A. (Eds.), *Animal Products from the Mediterranean Area – EAAP 119*. Wageningen Academic Publishers, The Netherlands, pp. 331–336.
- Morató, R., Romaguera, R., Izquierdo, D., Paramio, M.T., Mogas, T., 2011. Vitrification of *in vitro* produced goat blastocysts: effects of oocyte donor age and developmental stage. *Cryobiology* 63, 240–244.
- Papadopoulos, S., Rizos, D., Duffy, P., Wade, M., Quinn, K., Boland, M.P., Lonergan, P., 2002. Embryo survival and recipient pregnancy rates after transfer of fresh or vitrified, *in vivo* or *in vitro* produced ovine embryos. *Anim. Reprod. Sci.* 74, 35–44.
- Paria, B.C., Dey, S.K., 1990. Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors. *Proc. Natl. Acad. Sci. U. S. A.* 80, 4756–4760.
- Pereira, R.M., Mesquita, P., Batista, M., Baptista, M.C., Barbas, J.P., Pimenta, J., Santos, I.C., Marques, M.R., Vasques, M.I., Silva Pereira, M., Santos Silva, F., Oliveira Sousa, M.C., Fontes, C.M., Horta, A.E.M., Prates, J.A., Marques, C.C., 2009. Doppel gene polymorphisms in Portuguese sheep breeds: insights on ram fertility. *Anim. Reprod. Sci.* 114, 157–166.
- Pereira, R.M., Baptista, M.C., Vasques, M.I., Horta, A.E.M., Portugal, P.V., Bessa, R.J.B., Chagas e Silva, J., Silva Pereira, M., Marques, C.C., 2007. Cryo-survival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t, 12c CLA). *Anim. Reprod. Sci.* 98, 293–301.
- Pereira, R.M., Marques, C.C., 2008. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank* 9, 267–277.
- Peris, S.I., Morrier, A., Dufour, M., Bailey, J.L., 2004. Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. *J. Androl.* 25, 224–233.

PUBLISHED PAPERS

- Procházka, R., Petlach, M., Nagyová, E., Nemcová, L., 2011. Effect of epidermal growth factor-like peptides on pig cumulus cell expansion, oocyte maturation, and acquisition of developmental competence *in vitro*: comparison with gonadotropins. *Reproduction* 141, 425–435.
- Pugh, P.A., Fukui, Y., Tervit, H.R., Thompson, J.G., 1991. Developmental ability of *in vitro* matured sheep oocytes collected during the nonbreeding season and fertilized *in vitro* with frozen ram semen. *Theriogenology* 36, 771–778.
- Rizos, D., Ward, F., Duffy, P., Boland, M.P., Lonergan, P., 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* 61, 234–248.
- Sugiura, K., Pendola, F.L., Eppig, J.J., 2005. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev. Biol.* 279, 20–30.
- Thibier, M., Guérin, B., 2000. Embryo transfer in small ruminants: the method of choice for health control in germplasm exchanges. *Livest. Prod. Sci.* 62, 253–270.
- Uhm, S.J., Gupta, M.K., Yang, J.H., Chung, H.-J., Min, T.S., Lee, H.T., 2010. Epidermal growth factor can be used in lieu of follicle-stimulating hormone for nuclear maturation of porcine oocytes *in vitro*. *Theriogenology* 73, 1024–1036.
- Valente, S., Pereira, R.M., Baptista, M.C., Marques, C.C., Vasques, M.I., Silva Pereira, M.V.C., Horta, A.E.M., Barbas, J.P., 2010. *In vitro* and *in vivo* fertility of ram semen cryopreserved in different extenders. *Anim. Reprod. Sci.* 117, 74–77.
- van de Leemput, E.E., Vos, P.L., Zeinstra, E.C., Bevers, M.M., van der Weijden, G.C., Dieleman, S.J., 1999. Improved *in vitro* embryo development using *in vivo* matured oocytes from heifers superovulated with a controlled preovulatory LH surge. *Theriogenology* 52, 335–349.
- Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T., Callesen, H., 1998. Open pulled straws (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol. Reprod. Dev.* 51, 53–58.
- Wang, L.M., Feng, H.L., Ma, Y.Zh., Cang, M., Li, H.J., Yan, Zh., Zhou, P., Wen, J.X., Bou, S., Liu, D.J., 2009. Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. *Anim. Reprod. Sci.* 114, 99–108.

5 Papers submitted to publication

Ultrastructural characterization of fresh and vitrified *in vitro* and *in vivo* produced sheep embryos

Romão, R., Bettencourt, E., Pereira, R.M.L.N., Marques, C.C., Baptista, M.C., Barbas, J.P., Horta, A.E.M., Oliveira, E., Bettencourt, C., Sousa, M. Ultrastructural characterization of fresh and vitrified *in vitro* and *in vivo* produced sheep embryos. *Theriogenology* (submitted).

Cryopreservation of *in vitro* produced sheep embryos: effect of different protocols of lipid reduction

Romão, R., Marques, C.C., Baptista, M.C., Barbas, J.P., Horta, A.E.M., Carolino, N., Bettencourt, E., Pereira, R.M. Cryopreservation of *in vitro* produced sheep embryos: effect of different protocols of lipid reduction. *Theriogenology* (submitted).

Ultrastructure of *in vitro* produced sheep embryos: effect of *trans*-10 *cis*-12 conjugated linoleic acid (CLA) in fresh and cryopreserved blastocysts

Romão, R., Marques, C.C., Bettencourt, E., Baptista, M.C., Barbas, J.P., Carolino, N., Oliveira, E., Sousa, M., Pereira, R.M.L.N. Ultrastructure of *in vitro* produced sheep embryos: effect of *trans*-10 *cis*-12 conjugated linoleic acid (CLA) in fresh and cryopreserved blastocysts. *Animal* (submitted).

5.1 Ultrastructural characterization of fresh and vitrified *in vitro* and *in vivo* produced sheep embryos

Romão, R.^{1,2}, Bettencourt, E.¹, Pereira, R.M.L.N.^{2,3,4}, Marques, C.C.², Baptista, M.C.², Barbas, J.P.^{2,4}, Horta, A.E.M.², Oliveira, E.⁵, Bettencourt, C.⁶, Sousa, M.⁵

¹Escola de Ciências e Tecnologia, “ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas” - Universidade de Évora – Núcleo da Mitra, Ap. 94, 7002- Évora, Portugal

²Unidade de Recursos Genéticos, Reprodução e Melhoramento Animal, INRB IP, L INIA-Santarém, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal

³Escola Universitária Vasco da Gama, Mosteiro de S. Jorge de Milréu, Estrada da Conraria, 3040-714 Castelo Viegas, Coimbra, Portugal

⁴CIISA, Faculdade de Medicina Veterinária, Universidade de Lisboa Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal

⁵Department of Microscopy, Laboratory of Cell Biology, Multidisciplinary Unit for Biomedical Research-UMIB, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, 4050-313 Porto, Portugal

⁶Regional Agriculture Direction of Alentejo (DRAAL), Herdade da Abóbada, 7830-908 V.N.S. Bento, Portugal

Abstract

Cryopreservation of sheep embryo produced *in vitro* is a difficult procedure and present obtained results limits its routine use for research and commercial proposes when compared to *in vivo* produced embryos. Methods of *in vitro* embryo production are essentially derived from the ones used in cattle but results are still lower, so differences should be due to ovine embryo sensitivity, reason why it is necessary to find the mechanisms responsible for cellular damage induced by cryopreservation in this species. Although no ultrastructural differences have been observed between common freezing methods in *in vivo* sheep embryos, changes induced in *in vitro* sheep embryos by cryopreservation have not yet been presented. This work describes and compares, for the first time, ultrastructural changes in ovine blastocysts (BL) produced *in vitro* and cryopreserved in open pulled straws (OPS). Four groups of blastocysts were evaluated: fresh *in vitro* produced embryos (n=3), fresh *in vivo* produced embryos (n=3), warmed *in vitro* produced embryos cryopreserved by OPS (n=3) and warmed *in vivo* produced embryos cryopreserved by OPS (n=3). Ultrastructural observation of processed fresh embryos showed a reduced number of microvilli and mitochondria in the *in vitro* produced ones, appearing that those have also a lower number of mature mitochondria, with both having large number of vesicles, with light and dense content. These lower mitochondria content can be associated to deficient metabolism in *in vitro* produced embryos, that is possibly involved in the lower resistance to cryopreservation. In embryos vitrified by OPS major changes were observed mainly in *in vitro* produced embryos with small changes in grade 2 and high changes in grade 3 semithin scoring. The main changes associated to cryopreservation included disruption of cellular membranes and poor intracellular preservation, with loss of microvilli and presence of cellular debris. In conclusion, *in vitro* produced sheep embryos showed differences at ultrastructural level that can be associated to their lower resistance to cryopreservation and that can be related to metabolism and lipid content. Ultrastructural evaluation of cryopreserved *in vitro* produced blastocysts confirms the presence of more severe cellular damage in these embryos when compared to those produced *in vivo* and so future research in improving *in vitro* sheep embryo production and cryopreservation is needed to obtain acceptable and routine applicable results.

Keywords: ultrastructure; embryo; ovine; cryopreservation; *in vitro*

5.1.1 Introduction

Nowadays and in the future, *in vitro* embryo production is a biotechnological tool whose routine use is demanded for a wide range of applications. Although in some species *in vitro* embryo production is currently used, in small ruminants the associated high cost and inferior results, limits its commercial and even research application. The lower ability to develop and the greater sensibility to cryopreservation of *in vitro* produced embryos when compared to their *in vivo* counterparts ^[1,2,3,4,5], represent a big constrain for their storage limiting the routine use of this technique and the consequent effective genetic benefits. The improvement of cryopreservation techniques for sheep embryos is an important task not only for storage of genetic material from endangered breeds but also for future commercial purposes, being urgent to achieve competitive results to spread its use, as in other species.

Currently there are several methodologies that can be used to cryopreserve sheep embryos either *in vivo* or *in vitro* produced ^[4,5,6,7]. However, due to the huge sensitivity of *in vitro* embryos to chilling, better results have been achieved after cryopreservation by vitrification, and specifically by open pulled straw (OPS) ^[4,8,9]. This chilling sensitivity of *in vitro* produced embryos has been associated to differences in their metabolism ^[11] and ultrastructure ^[11,12,13,14]. In fact, *in vitro* produced embryos present several ultrastructural and biochemical characteristics that can reduce their resistance to cryopreservation, namely a greater abundance of lipid, a reduced number of mitochondria and microvilli, a reduced cell network of intercellular junctions, a larger perivitelline space and a higher incidence of cellular debris, than their *in vivo* counterparts ^[11,12,13,14,15,16,17,18,19]. Moreover the cryopreservation process causes several additional changes in embryo ultrastructure ^[2,20,21] that have been associated with the lower viability of cryopreserved embryos ^[20].

The ultrastructure of *in vivo* produced sheep embryos, either fresh or cryopreserved, has been previously described ^[17,21,22], but no reports comparing the effect of OPS cryopreservation in the ultrastructure of *in vitro* and *in vivo* produced sheep embryos were performed. The objective of this study was to evaluate the effect of the cryopreservation by OPS in the ultrastructure of *in vitro* produced sheep embryos comparing with their *in vivo* counterparts.

5.1.2 Materials and methods

All chemicals used were purchased from Sigma Aldrich Chemical Co. (St. Louis, USA) unless specified otherwise.

5.1.2.1 *In vivo* embryo production

In vivo produced embryos (n=6) were obtained from Merino ewes, superovulated during spring. Donors were synchronized with intravaginal sponges containing 40 mg fluorogestone acetate (Chronogest, Intervet Laboratories, Boxmeer, Holand) for 12 days. Starting on day 9 of sponge treatment and for 4 consecutive days, ewes were injected (im) with 1.25 ml ovine FSH (oFSH; Ovagen, Immunological Products Ltd, Auckland, New Zealand), twice a day time (9:00 and 21:00h). Sponges were removed at the time of the 7th oFSH administration. Laparoscopic intrauterine insemination was performed with fresh diluted semen in all ewes, 48 hours after sponge removal, as described previously ^[23]. A minimum of 50×10^6 motile spermatozoa was placed in each uterine horn. Additionally, ewes were hand mated at 36 h and 48 h after sponge withdrawal and left with rams for 4-6 h at a ewe:ram ratio of 3:1. On day 6 or 7 after sponge removal, embryos were recovered by abdominal laparotomy and uterine wash under general anaesthesia. Each uterine horn was flushed using a Foley catheter and 40 ml of collection medium (PBS containing 2% bovine serum albumin – BSA) as in Bettencourt *et al.* ^[21].

5.1.2.2 *In vitro* embryo production

Ovine ovaries were collected at a local slaughterhouse and transported to the laboratory in Dulbecco's phosphate buffer saline (PBS, GibCo 14040-91) at 35°C. PBS was supplemented with 0.15% (w/v) of BSA and 0.05 mg mL⁻¹ of kanamycin. At the laboratory, 2-6 mm follicles were aspirated to obtain the cumulus oocyte complexes (COC) and selected COCs were matured for 22h at 38.5°C and 5% CO₂ in 4-well dishes (Nunc, Nunclon, Denmark) with each well containing 20–30 oocytes covered with mineral oil. The maturation medium consisted of Tissue Culture Medium 199 (M199), 10 µM cysteamine, 0.3 mM sodium pyruvate, 10 µg mL⁻¹ FSH, 10 ng mL⁻¹ EGF, 10 µg mL⁻¹ estradiol and 10 µL mL⁻¹ gentamicin (Romão *et al.*, 2013).

Fresh semen from a Merino breed ram of proven fertility was used throughout the experiments. Collected ejaculates were kept at room temperature and light protected for up to 2 h, then washed in synthetic oviductal fluid (SOF) and centrifuged at 225Xg for 5 min. This medium was SOF enriched with 4 mg mL⁻¹ BSA plus 1.5 µg mL⁻¹ glutamine and kept at the same room temperature. Subsequently washed spermatozoa were diluted with 1 mL of fertilization medium consisting of SOF containing 20 µL mL⁻¹ BME Amino Acids Solution (BME) and 10 µL mL⁻¹ MEM Amino Acids Solution (MEM), 10 µL mL⁻¹ gentamicin and 10% ovine oestrus serum. IVF was performed as in Pereira *et al.* ^[24] using 1×10^6 spz mL⁻¹. Eighteen hours after insemination, presumptive zygotes were transferred into droplets of SOF supplemented with BME and MEM amino acids, 6 mg mL⁻¹ BSA. After assessing cleavage, embryo development proceeded until the

blastocyst stage in amino acids and BSA supplemented SOF at the some doses as above, plus 10% fetal calf serum (SOFserum). Embryo culture was performed at 38.5°C in a humidified atmosphere with 5% O₂, 5% CO₂ and 90% N₂. Cleavage rate was calculated as the number of cleaved embryos per number of inseminated oocytes. Day 6/7 embryo developmental rate was calculated as the number of D6/7 blastocysts per number of cleaved embryos.

5.1.2.3 Embryo evaluation and selection

Morphological scoring of sheep embryos was performed based on morphological criteria and developmental stage in accordance to International Embryo Transfer Society guidelines^[23]. Embryos, at the stage of morulae or blastocyst (BL) were scored as grade 1 (excellent or good) when presenting a symmetrical and spherical cell mass with ≥75% of intact cells (<25% of degenerated cells/cell fragments), with individual cells showing an uniform size, colour and density and grade 2 embryos (fair) had a cell mass with ≥50% of intact cells (25-50% of degenerated cells/cell fragments), with individual cells presenting moderate irregularities in size, colour and density.

Only young blastocysts of grade 1 were selected for freezing. Additionally fresh *in vivo* (n=3) and *in vitro* (n=3) produced young blastocysts (control *in vivo* and *in vitro* groups, respectively) classified as grade 1 were directly processed for transmission electron microscopy.

5.1.2.4 Embryo cryopreservation by open pulled straw (OPS)

All embryos were cryopreserved by vitrification using the OPS method as described by Vajta *et al.*^[24]. Briefly, embryos were first equilibrated in holding media (HM: TCM199 plus 20% bovine serum) for 5 min, then in diluted (HM with 10% ethylene glycol–EG and 10% DMSO, 1 min) and afterwards in concentrated (HM with 20% EG and 20% DMSO) vitrification media. Embryos were quickly placed into the superfine OPS and plunged directly into liquid nitrogen (NL₂, 30 sec).

5.1.2.5 Warming and embryo scoring

Embryo warming was performed by placing the end of the straw directly into the HM at 38.5°C. After 2 min, blastocysts were transferred into HM for another 2 min; and then to SOFserum and cultured for 3 hours as in Romão *et al.*^[27]. Morphological scoring of embryos was performed based on morphological criteria and developmental stage in accordance to International Embryo Transfer Society guidelines as above^[25].

5.1.2.6 Electron microscopy processing

Fresh *in vivo* (n=3) and *in vitro* (n=3) produced BL and cryopreserved *in vivo* (n=3) and *in vitro* (n=3) produced BL were fixed in Karnowsky medium (2 h, 4°C), washed with 0.15 M sodium cacodylate buffer, pH 7.3 (overnight, 4°C), post-fixed (1 h, 4°C) in 1% OsO₄ in buffer containing 0.8% hexanocyanoferrate potassium [K₃Fe³⁺(CN)₆], and washed for 15 min in buffer. They were then dehydrated through a graded series of ethanol (50, 70, 90, 2x100%; 30 min each, at room temperature), followed by propylene oxide (15 min), impregnated with propylene oxide:Epon (3:1, 1 h; 1:1, at room temperature, overnight, 4°C; 1:3, 4 hours at room temperature) and embedded in Epon (7 h at room temperature, 3 days at 60°C). Sections were cut in a Leica ultramicrotome with a Diatome knife. Semithin sections were stained with aqueous azur II:methylene blue (1:1). Ultrathin sections were collected on 200 mesh copper grids (Taab) and stained with 3% aqueous uranyl acetate (20 min) and Reynolds lead citrate (10 min). Sections were observed in a transmission electron microscope JEOL 100CXII operated at 60kV.

5.1.3 Results

Grade 1 fresh *in vitro* and *in vivo* produced blastocysts were fixed immediately and then were rescored on semithin sections according to the International Embryo Transfer Society guidelines^[25]. Semithin sections of vitrified embryos, processed one hour after warming, were also rescored. Most of *in vitro* produced OPS vitrified blastocysts were scored as grade 2 and 3 at semithin section, comparing with *in vivo* produced OPS vitrified embryos that were all scored as grade 1 and 2.

At the ultrastructural level no main differences were observed in fresh embryos between trophoblast and inner cell mass (ICM) cells in both *in vitro* (figure3) and *in vivo* produced blastocysts (figure 4), with the exception of the presence of microvilli in the outer, trophoblast cells (figures 3A, 3C, 4A, 4C). Despite in some trophoblast cells of *in vitro* produced blastocysts numerous microvilli could be observed (figure 3A) microvilli were more abundant in *in vivo* produced embryos (figure, 4A, 4C). Most cells of fresh embryos presented intact membranes and well preserved cytoplasm and a well-defined and organized nucleus, with an intact nuclear envelope and one or more reticular nucleolus (figures 3 and 4). Mitochondria displaying peripheral and transverse cristae, frequently associated to cisternae of smooth endoplasmic reticulum, appeared more abundant at *in vivo* produced embryos. Moreover immature mitochondria with fewer peripheral cristae were apparently the most frequent type observed in *in vitro* produced embryos. Smooth endoplasmic reticulum and Golgi complexes appeared also more abundant at *in vivo* embryos than *in vitro*

ones (fig 3 and 4). Small and large light vesicles, as well, as medium and large dense vesicles were seen in both *in vitro* and *in vivo* BL. Light vesicles fused each other's (figures 3 and 4).

OPS vitrified *in vitro* produced BL, classified as grade 2 and 3 in semithin scoring, presented different grades of cell damage. Grade 2 vitrified *in vitro* BL (figure 5), despite presenting some well-preserved cells, also showed some degenerated cells. Well-preserved cells (figure 5A, 5B) exhibited intact membranes and well-maintained cytoplasm, with organized and well defined nucleus and nucleolus. Large light vesicles were also seen. Dense vesicles were not observed at vitrified embryos. Degenerated cells presented signs of deterioration namely disruption of nuclear membrane and poor intracellular preservation (figure 5C, 5D). In grade 3 OPS vitrified *in vitro* BL (figure 5C, 5D), there were additional degenerative alterations namely loss of microvilli, non-defined cellular limits, poor cellular preservation and presence of cellular debris and cellular lysis (figure 5E, 5F). OPS vitrified *in vivo* produced BL scored as grade 1 showed well preserved cytoplasm presenting the same organelles described for fresh embryos. Intercellular junctions (fig. 6A) and a well-defined nucleus and nucleolus were also observed (Fig. 6B, 6C). OPS vitrified *in vivo* BL scored as grade 2 showed some signs of degeneration, namely increased cell debris and reduced number of microvilli (Fig. 6C, 6D).

5.1.4 Discussion

In vitro produced embryos present a greater sensitivity to cryopreservation and this has been associated with differences in their ultrastructure ^[11,12,13,14]. Previous reports described the ultrastructure of fresh and cryopreserved cattle *in vitro* produced embryos ^[18,21] and sheep fresh and cryopreserved *in vivo* produced embryos ^[21,28,29], but, as far as our knowledge, no study compares the ultrastructure of *in vitro* and *in vivo* sheep embryos, either fresh or vitrified by OPS.

In what concerns fresh embryos, in the current study the differences observed between *in vivo* and *in vitro* sheep blastocysts are similar to what was previously described for cattle, namely a reduced number of microvilli and mitochondria in the last group ^[12,17]. Microvilli are cytoplasmatic extensions projected into the perivitelline space that augment the external surface of the blastocyst ^[16] increasing its absorption capabilities ^[30]. Although the reduction in the number of microvilli observed in *in vitro* produced embryos was hypothetically associated with poor absorption capabilities, no differences were observed in the viability between *in vitro* embryos with reduced number of microvilli and their *in vivo* counterparts ^[31].

Despite the quantification of neither the total, nor mature or immature mitochondria was done, it appears that *in vitro* produced embryos presented less number of mitochondria than *in vivo* embryos. This

fact was also previously described by Crosier *et al.* ^[12]. In fact the reduced number of mitochondria as well as the reduced number of mature mitochondria has been associated to an inefficient metabolism of lipids observed in *in vitro* produced embryos ^[12,32]. The degeneration of mitochondria observed in these latter embryos was associated with the presence of serum in the SOF ^[33]. Also Dalcin *et al.* ^[7] referred a decrease in mitochondrial activity in sheep vitrified embryos when compared with fresh ones. The influence of the proportion of mitochondria in lipid content was also proposed by Crosier *et al.* ^[12] who observed a reduction in the volume density of mature mitochondria in *in vitro* embryos developed in three different culture media, even in the absence of serum. Therefore the increased embryo lipid content can result from membrane breakdown in response to a non-physiological culture environment and/or from the fatty acid uptake of the serum containing media ^[12,33]. Moreover the presence of large number of vesicles, with light and dense contents was observed in fresh *in vitro* but also *in vivo* produced embryos as previously described ^[21,22,29]. Despite they had not been estimated in this study, apparently the presence of a large number of lipid droplets was higher in *in vitro* produced sheep embryos.

In the present study, OPS vitrified *in vitro* produced BL presented more severe signs of degeneration than *in vivo* produced ones. These alterations vary from small changes in grade 2 BL at semithin score to large alterations in grade 3 BL. Moreover the differences among cells observed in grade 2 embryos can be associated to different rates of penetration of cryoprotectors into cells as well as to the reduction of function of cytoskeleton and intercellular junctions in the *in vitro* embryos ^[15]. The main alterations associated to cryopreservation included disruption of cellular membranes and poor intracellular preservation. As previous described by Crosier *et al.* ^[12] and Rizos *et al.* ^[17] the lower number of microvilli, less extensive network of intercellular junctions, associated with the presence of more cellular debris and reduced number of mitochondria could contribute to the reduced cryotolerance of *in vitro* produced embryos. In porcine BL vitrification also caused ultrastructural changes such as accumulation of cellular debris, an increase of vesicles, vacuoles and lysosomes ^[34,35]. In cryopreserved cattle embryos, Ohboshi *et al.* ^[20] reported damage to the membrane functions by observation of disruption of the plasma membrane, changes in the mitochondrial cristae and matrix, and swelling of rough endoplasmic reticulum. They also observed a decrease in microvilli that indicated difficulties in further embryo development as these structures are important for fluid transport activity in the BL ^[30]. Moreover the higher degree of cell damage with complete cell lysis, disruption of cytoplasmic and nuclear membranes can also be associated with severe mitochondrial damage ^[21,28] and impaired lipid metabolism ^[36,37]. This negative association may indicate that

the increase in mitochondrial area enhances the ability to perform embryonic β -oxidation of fatty acids and that on the contrary impaired mitochondria and lipid accumulation are detrimental for embryo development [12,38]. In fact mitochondria metabolize fatty acids via the β -oxidation pathway to generate cellular ATP essential for embryonic development [39]. Likewise Crocco *et al.* [38] reported a negative association between the area covered by lipid droplets and hooded mitochondria in cattle embryos. Lipid droplets are the main storage of triacylglycerols in the embryo [33,37] and are present in high content in *in vitro* produced BL. This high lipid content is often related to an increased sensitivity to chilling injury during cryopreservation [19,36]. Lipid droplets interact directly with the intermediate filaments of the cytoskeleton and physical changes within these organelles during cryopreservation may lead to irreversible damages in the cytoskeleton [40,41]. These observations strongly suggest that embryo development ability and cryotolerance are undoubtedly related to lipid metabolism and thus to lipid droplets dynamics and properties.

In conclusion *in vitro* produced sheep embryos showed differences at ultrastructural level that can be associated to their lower resistance to cryopreservation. Ultrastructural evaluation of cryopreserved (OPS) *in vitro* produced BL confirms the presence of more severe cellular damage in these embryos when compared to those produced *in vivo*. These ultrastructural alterations are associated with degeneration of the various components of the cell and can impair further embryo development. So they are probably responsible for the lower pregnancy rates achieved after transfer of *in vitro* cryopreserved sheep embryos. Additional research is needed to allow the development of the *in vitro* embryo production protocols. An alternative could be the application of different treatments, namely reducing lipid content of the *in vitro* produced embryos previously to their cryopreservation.

5.1.5 References

- [1] Massip A, Mermillod P, Dinnyes A. Morphology and biochemistry of *in-vitro* produced bovine embryos: implications for their cryopreservation. Hum. Reprod. 1995;10(11):3004-3011 (abstr).
- [2] Farin, PW, Crosier AE, Farin CE. Influence of *in vitro* systems on embryo survival and fetal development in cattle. Theriogenology 2001;55: 151-170.
- [3] Dieleman SJ, Hendriksen PJM, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TAM, Niemann H, Gadella BM, Bevers MM, Vos PLAM. Effect of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology 2002;57,5-20.
- [4] Dattena M, Accardo C, Pilchi S, Isachenko V, Mara L, Chessa B, Cappai P. Comparison of different vitrification protocols on viability after transfer of ovine blastocysts *in vitro* produced and *in vivo* derived. Theriogenology 2004;62:481-493.
- [5] Martínez AG, Valcácel A, Furnus CC, de Matos DG, Iorio, G, de las Heras MA. Cryopreservation of *in vitro*-produced ovine embryos. Small Rum Res 2006;63(3):288-296.

- [6] Dattena M, Ptak G, Loi, P, Cappai P. Survival and viability of vitrified *in vitro* and *in vivo* produced ovine blastocysts. *Theriogenology* 2000;53:1511-1519.
- [7] Dalcin L, Silva RC, Paulini F, Silva BD, Neves JP, Lucci CM. Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos. *Cryobiology* 2013;67(2):137-45.
- [8] Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reproductive Bio-Medicine Online* 2006;12:779–96.
- [9] Vajta G, Kuwayama M. Improving cyopreservation systems. *Theriogenology* 2006;65:236-44.
- [10] Khurana NK, Niemann H. Effects of cryopreservation on glucose metabolism and survival of bovine morulae and blastocyst derived *in vitro* or *in vivo*. *Theriogenology* 2000;54:313-326.
- [11] Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H. Ultrastructure of bovine embryos developed from *in vitro*-matured and –fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. *Molecular Reproduction and Development* 1999;53:325-335.
- [12] Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine compact morulae produced *in vivo* or *in vitro*. *Biology of Reproduction* 2000;62:1459-1465.
- [13] Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine blastocysts produced *in vivo* or *in vitro*. *Biology of Reproduction* 2001;64:1375-1385.
- [14] Fair T, Lonergan P, Dinnyes A, Cottell D, Hyttel P, Ward FA, Boland MP. Ultrastructure of bovine blastocysts following cryopreservation: effect of method of embryo production on blastocyst quality. *Mol Reprod Dev* 2001;58:186-195.
- [15] Boni R, Tosti E, Roviello S, Dale B. Intercellular communication in *in vivo*- and *in vitro*-produced bovine embryos. *Biology of Reproduction* 1999;61:1050-1055.
- [16] Abe H, Matsuzaki, S, Hoshi H. Ultrastructural differences in bovine morulae classified as high and low qualities by morphological evaluation. *Theriogenology* 2002;57:1273-1283.
- [17] Rizos, D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* 2002;61:234-248.
- [18] Seider Jr GE. Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology* 2006;65:228-235.
- [19] Pereira RM, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Chagas e Silva J, Silva Pereira M, Marques CC. Cryo-survival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t,12c CLA). *Animal Reproduction Science* 2007;98:293-301.
- [20] Ohboshi S, Fujihara N, Yoshida T, Tomagane H. Ultrastructure of bovine *in vitro*-produced blastocysts cryopreserved by vitrification; *Zygote* 1998;6:17-26.
- [21] Bettencourt EMV, Bettencourt CM, Silva JNCE, Ferreira P, de Matos CP, Oliveira E, Romão RJ, Rocha A, Sousa M. Ultrastructural characterization of fresh and cryopreserved *in vivo* produced ovine embryos. *Theriogenology* 2009;71(6):947-58.
- [22] Ferrer F, Garcia C, Villar J, Arias M. Ultrastructural study of the early development of the sheep embryo. *Anat Histol Embryol* 1995;24(3):191-6.
- [23] McKelvey AC, Robinson JJ, Aitken RP, Henderson G. The evaluation of a laparoscopic insemination technique in ewes. *Theriogenology* 1985;24:519-35.
- [24] Pereira, RM, Mesquita P, Batista M, Baptista MC, Barbas JP, Pimenta J, Santos IC, Marques MR, Vasques MI, Silva Pereira M, Santos Silva F, Oliveira Sousa MC, Fontes, CM, Horta AEM, Prates JA, Marques CC. Doppel gene polymorphisms in Portuguese sheep breeds: insights on ram fertility. *Anim. Reprod. Sci.* 2009;114:157-166.
- [25] Stringfellow DA, Seidel SM, editors. *Manual of the International Embryo Transfer Society Savoy II, USA; 1998.*

PAPERS SUBMITTED TO PUBLICATION

- [26] Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open pulled straws (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development* 1998;1:53-58.
- [27] Romão R, Marques CC, Baptista MC, Vasques MI, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Plancha C, Rodrigues P, Pereira RM. Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen. *Small Ruminant Research* 2013;110:36-41.
- [28] Cocero MJ, Moreno Díaz de la Espina S, Aguilar B. Ultrastructural characteristics of fresh and frozen-thawed ovine embryos using two cryoprotectants. *Biology of Reproduction* 2002;66:1244-1258.
- [29] Bettencourt EMV, Bettencourt CMV, Ferreira P, Chagas da Silva J, Oliveira E, R Romão, Rocha A, Sousa M. Ultrastructural study of *in vivo* produced ovine embryos: characterization of secretory vesicles. *Reprod Dom Anim* 2013;48(Suppl.1):104.
- [30] Betteridge KJ, Flechon J-E. The anatomy and physiology of pre-attachment bovine embryos. *Theriogenology* 1988;29:155-187.
- [31] Thompson JG. Defining the requirements for bovine embryo culture. *Theriogenology* 1996; 45:27-40.
- [32] Dorland M, Gardiner DK, Trounson AO. Serum in synthetic oviduct fluid causes mitochondrial degeneration in ovine embryos. *J Reprod Fertil* 1994;13:25 (abstr).
- [33] Lapa M, Marques CC, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Pereira RM. Fatty acid profile of oocytes and blastocysts during *in vitro* bovine embryo production. *Reproduction in Domestic Animals* 2005;40(4):365.
- [34] Fabian D, Gjørret JO, Berthelot F, Martinat-Botte F, Maddox-Hyttel P. Ultrastructure and cell death of *in vivo* derived and vitrified porcine blastocysts. *Molecular Reproduction and Development* 2005;70:155–165.
- [35] Cuello C, Berthelot F, Delaleu B, Venturi E, Pastor LM, Vazquez JM, Roca J, Martinat-Botte F, Martinez EA. The effectiveness of the stereomicroscopic evaluation of embryo quality in vitrified–warmed porcine blastocysts: an ultrastructural and cell death study. *Theriogenology* 2007;67:970–982.
- [36] Abe H, Shiku H, Aoyagi S, Hoshi H. *In vitro* culture and evaluation of embryos for production of high quality bovine embryos. *J. Mamm. Ova Res.* 2004;21:22-30.
- [37] Prates EG, Nunes JT, Pereira RMLN. A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production. *Mediators of Inflammation* 2014, Article ID 692067:1-11. <http://dx.doi.org/10.1155/2014/692067>
- [38] Crocco MC, Kelmansky DM, Mariano MI. Does serum cause lipid-droplet accumulation in bovine embryos produced *in vitro*, during developmental days 1 to 4? *J Assist Reprod Genet* 2013;30(10):1377-88.
- [39] Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol Reprod* 2010;83(6):909-18.
- [40] Pereira RM, Marques CC. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank* 2008;9:267-277.
- [41] Zehmer JK, Huang Y, Peng G, Pu J, Anderson RG, Wand Liu P. A role for lipid droplets in inter-membrane lipid traffic. *Proteomics* 2009;9:914-921.

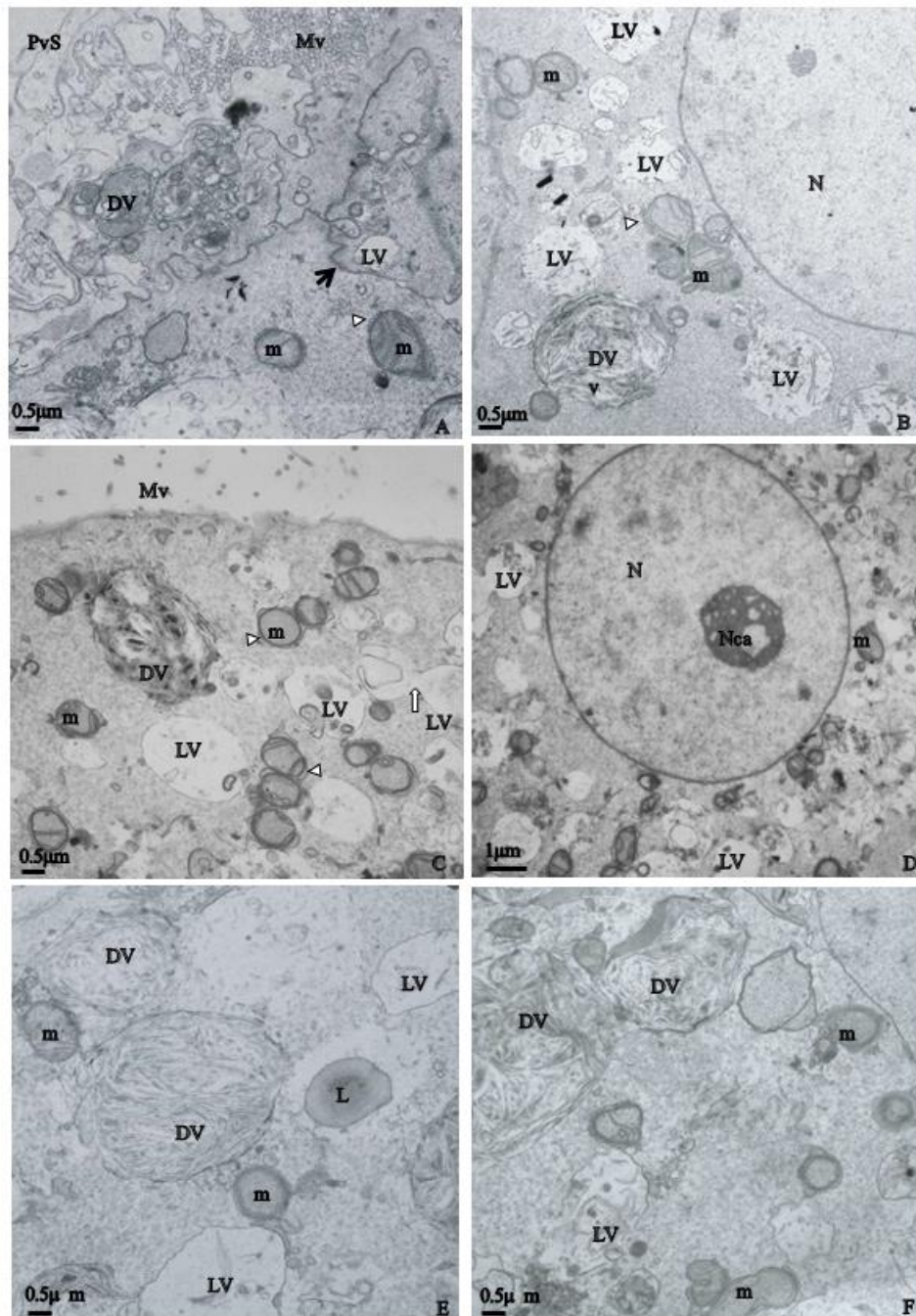


Figure 3: Ultrastructure of fresh *in vitro* ovine embryos at early blastocyst stage. Outer (A,C,E) and inner cells (B,D,F), cells. Microvilli (Mv), perivitelline space (PvS), cell debris (*), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), lipid droplets (L), fusion (white open arrow) of light (LV) and dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc).

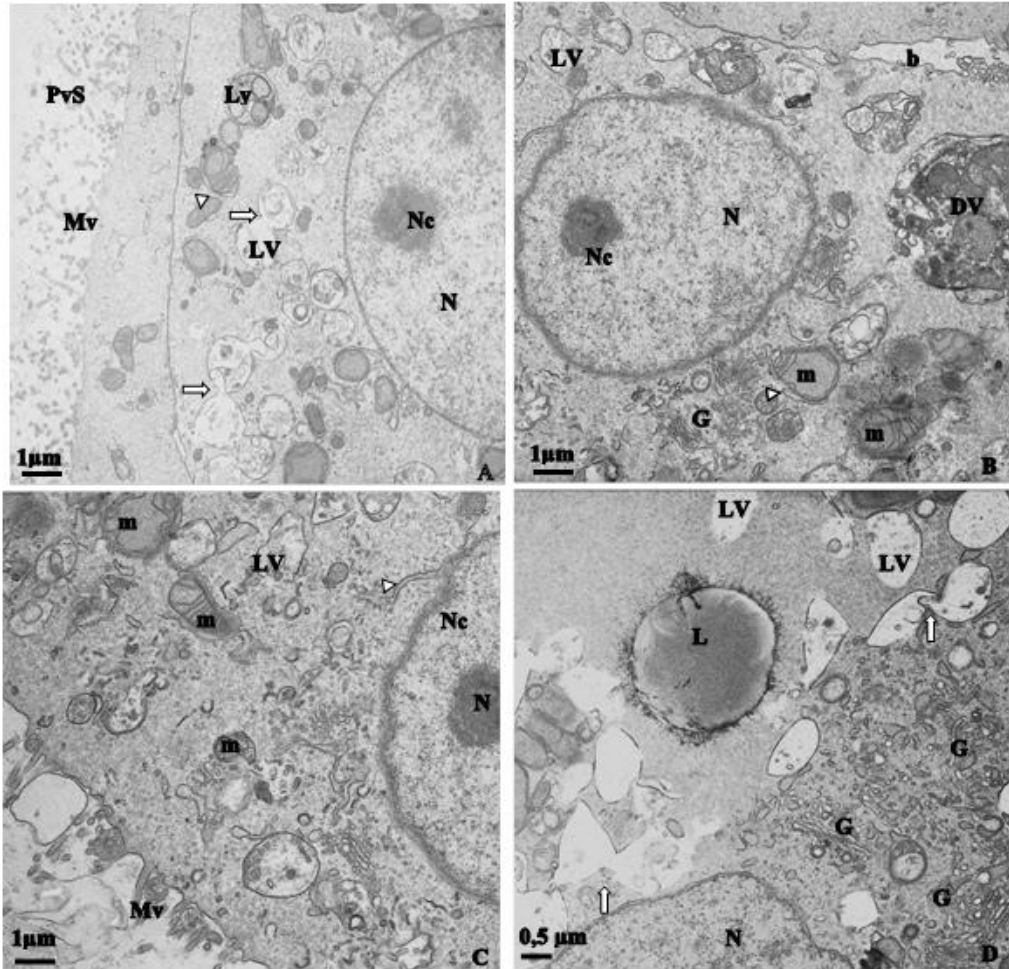


Figure 4: Ultrastructure of fresh *in vivo* ovine embryos at early blastocyst stage. Outer (A,C) and inner cells (B,D), cells. Microvilli (Mv), perivitelline space (PvS); fusion of light vesicles (white arrow), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), rough endoplasmic reticulum (black arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), lipid droplets (L), secondary lysosomes (Ly).

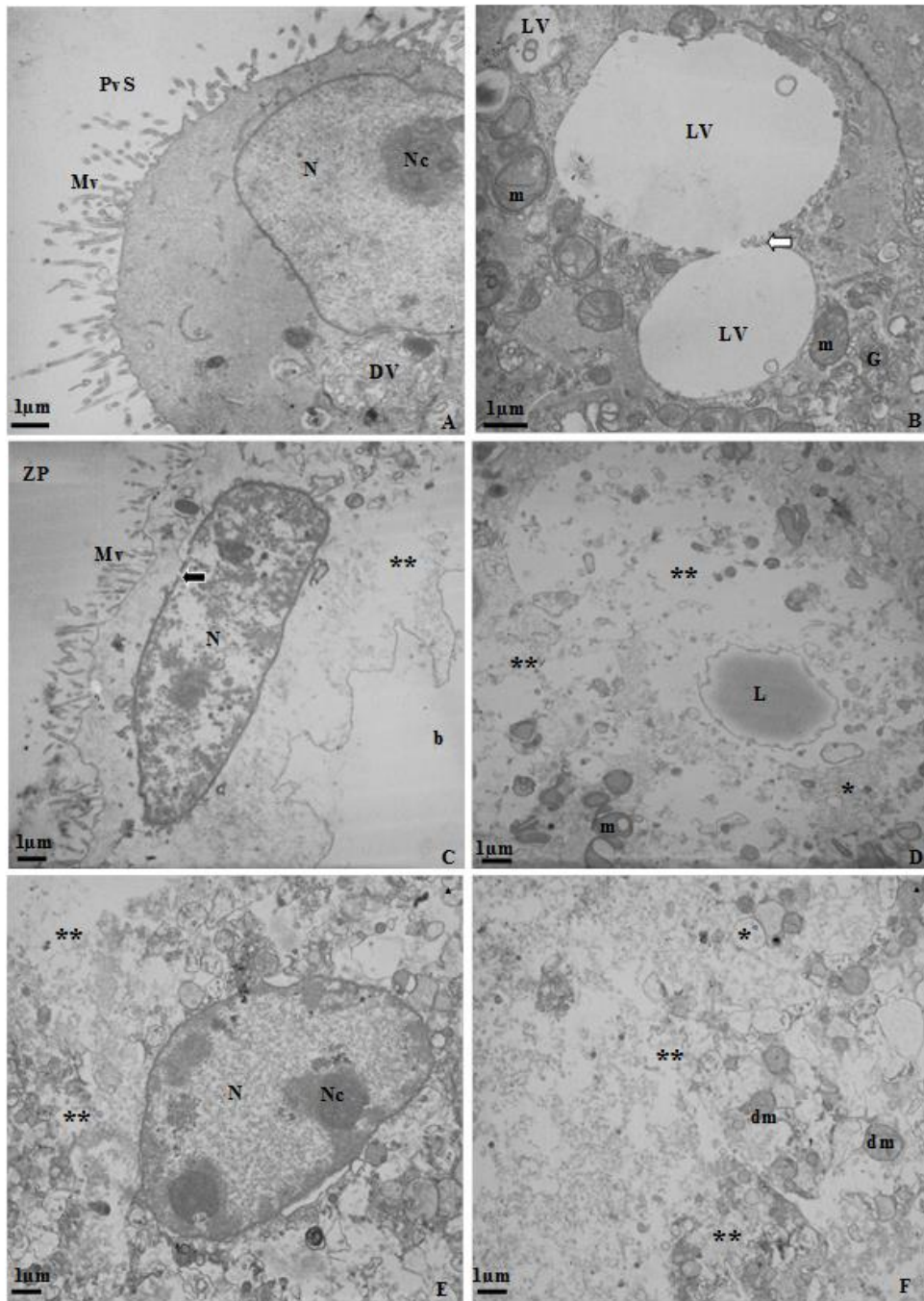


Figure 5: Ultrastructure of OPS vitrified *in vitro* sheep embryos at early blastocyst stage fixed 1h after thawing. Blastocysts were classified as grade 2 (A and B) and grade 3 (C and D) in semithin sections. Zona pellucida (ZP) perivitelline space (PvS), microvilli (Mv), blastocoelic cavity (b), nuclear membrane disruption (black open arrow), mitochondria (m), degenerated mitochondria (dm), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), fusion of light vesicles (white open arrow), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), cell debris (*), cell lysis (**)

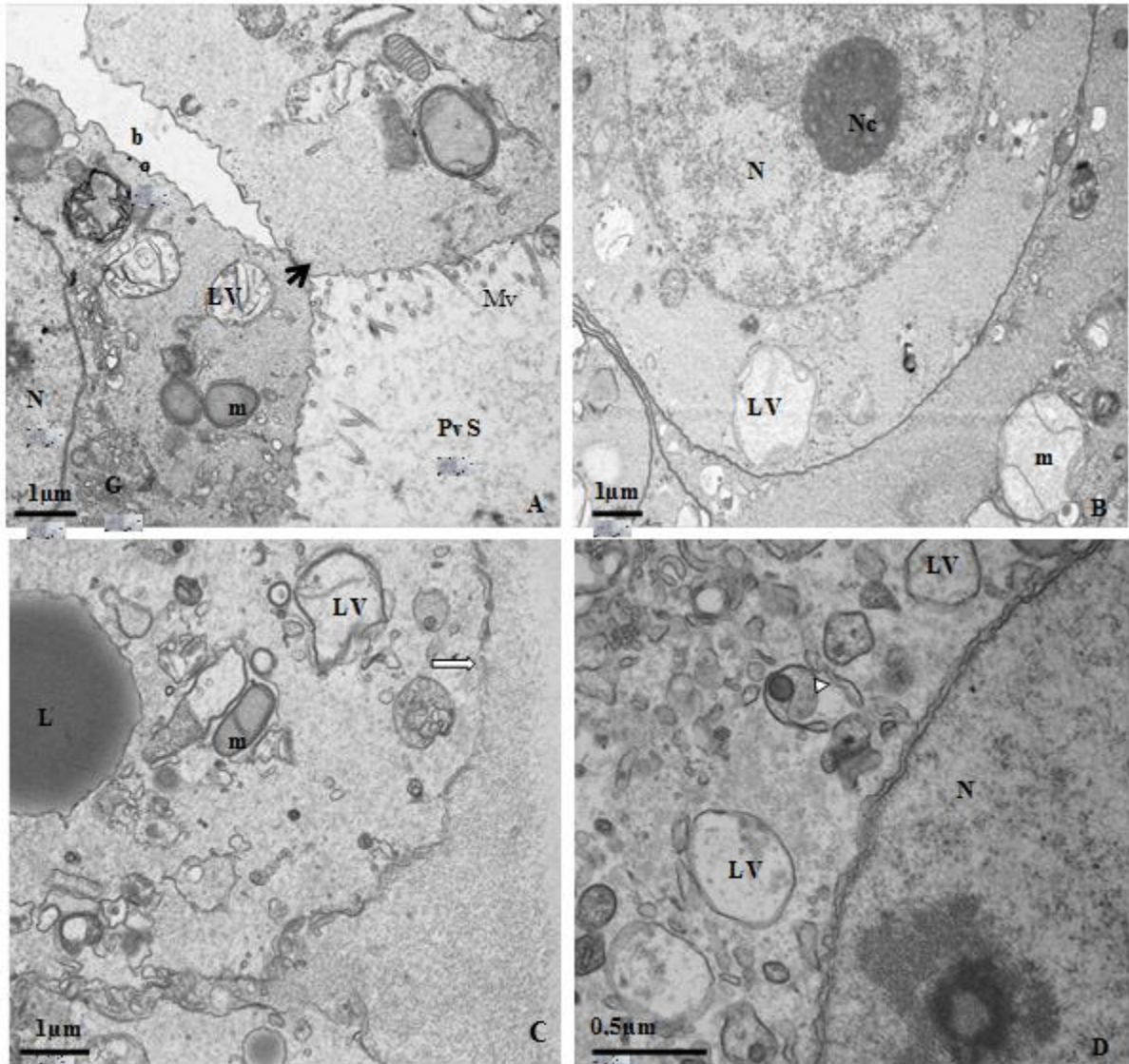


Figure 6: Ultrastructure of OPS vitrified *in vivo* ovine embryos at early blastocyst stage fixed 1h after thawing. Blastocysts were classified as grade 1 (A and B) and grade 2 (C and D) in semithin sections. Perivitelline space (PvS), microvilli (Mv), blastocoelic cavity (b), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), lipid droplets (L).

5.2 Cryopreservation of *in vitro* produced sheep embryos: effect of different protocols of lipid reduction

Romão, R.^{1,2}, Marques, C.C.¹, Baptista, M.C.¹, Barbas, J.P.^{1,3}, Horta, A.E.M.¹, Carolino, N.^{1,3,4}, Bettencourt, E.², Pereira, R.M.^{1,3,4}

¹Unidade de Biotecnologia e Recursos Genéticos, INIAV-Santarém, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal

²Escola de Ciências e Tecnologia, “ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas” - Universidade de Évora – Núcleo da Mitra, Ap. 94, 7002- Évora, Portugal

³CIISA, Universidade de Lisboa Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal

⁴Escola Universitária Vasco da Gama, Av. José R. Sousa Fernandes, Campus Universitário – Bloco B, Lordemão, 3020-210 Coimbra, Portugal

Abstract

The low survival of sheep *in vitro* produced (IVP) embryos after cryopreservation is a key limiting step to the widespread of this technology. In the present work, different approaches for enhancing cryosurvival of these embryos were compared: embryo delipidation by centrifugation in the absence or presence of cytochalasin D, a cytoskeleton stabilizer, or by embryo culture in the presence of different doses of the *trans*-10 *cis*-12 conjugated linoleic acid isomer (CLA). Three experiments were conducted. In exp.1, IVP blastocysts prior to vitrification were randomly distributed into 4 groups: control; centrifuged (cent); cytochalasin D (cyto-D); centrifuged+cytochalasin D (cent+cyto-D). In exp. 2, different doses of CLA (25, 50 and 100 μ M) were supplemented during embryo culture prior to blastocysts vitrification. A control group ran simultaneously. A third experiment was performed to compare both approaches from the previous ones but without the groups with the worst results (groups: control, cyto-D, cent+cyto-D, CLA25, CLA50). In all experiments, embryos integrity and re-expansion were assessed post-warming and after 3 hours of culture. In exp. 1, the post warming integrity rate was the lowest ($p<0.05$) in embryos from cent group (cent: $50.6\pm 10.3\%$ vs. control: $74.6\pm 9.2\%$, cyto-D $92.3\pm 9.7\%$ and cent+cyto-D $90.5\pm 11.2\%$), while the best ($p<0.05$) re-expansion scores were obtained in cent+cyto-D embryos (cent+cyto-D: 2.6 ± 0.28 vs. control: 1.8 ± 0.08 , cent: 1.9 ± 0.2 and cyto-D: 1.8 ± 0.31). In exp. 2 and 3, higher ($p<0.05$) cleavage rates were observed in CLA25 ($50.9\pm 6.2\%$ and $49.2\pm 5.6\%$, respectively) and CLA50 ($48.9\pm 6.2\%$ and $47.6\pm 5.6\%$, respectively) than in control ($41.8\pm 6.1\%$ and $40.4\pm 5.4\%$, respectively) group. In exp. 2, CLA100 presented the lowest ($p<0.002$) D6/7 embryo production rate and quality. After warming superior ($p<0.02$) expansion scores were achieved in CLA25 (3.1 ± 0.29) and CLA50 (3.8 ± 0.17) than in control (1.9 ± 0.10) group. Similar results were attained in exp. 3. However although cent+cyto-D embryos showed higher ($p=0.008$) post warming expansion scores than control (2.8 ± 0.29 vs. 1.9 ± 0.07), this score was lower ($p=0.0009$) than in CLA50 embryos (3.8 ± 0.17). In conclusion our results showed that different protocols of lipid reduction can be successfully applied to improve the cryotolerance of IVP sheep embryos.

Keywords: sheep embryos; lipid reduction, CLA, cytochalasin; ultracentrifugation, cryopreservation.

5.2.1 Introduction

The cryopreservation of *in vitro* produced sheep embryos is a biotechnological tool that still achieves unsatisfactory results. Although acceptable embryo production rates can be obtained in sheep (cleavage rate: 55-83%; blastocysts rate: 27-53%), only 10-19.4% of lambs are born after transferring frozen-thawed *in vitro* produced (IVP) embryos ^[1,2,3,4]. Conversely significantly higher pregnancy and lambing rates can be achieved of recipients bearing *in vivo* derived cryopreserved embryos (50-70.3 and 50-62.9% respectively ^[2,5]).

Many factors contribute to the low viability observed in cryopreserved preimplantation embryos produced by *in vitro* culture techniques. According to several authors, the reduced quality of IVP embryos can be associated with oocyte quality but also with the composition of maturation medium ^[1,3,6,7]. Moreover the quality and status (fresh or cryopreserved) of semen used for *in vitro* fertilization is also important. Recent reports showed that oocyte fertilized by fresh semen clearly improved sheep embryo developmental competence by enhancing morphological blastocyst quality ^[7]. Notwithstanding an increased lipid accumulation in IVP embryos has also been correlated to a reduced cryotolerance leading to lower pregnancy success ^[2,8,9,10,11]. This higher level of intracellular lipid content depends on culture conditions, being increased by serum containing media ^[8,10,12]. Serum is useful in oocyte and embryo culture as a source of albumin that balances the osmolarity, acting as a free radical scavenger with an additional important nutritive role ^[13]. However, the fatty acids and lipoproteins of the serum seem to be the source of embryos' cytoplasmic lipids, hampering embryo quality ^[14,15], albeit the perturbations induced by the presence of serum in sheep embryo culture are higher before rather than after compaction ^[16].

Different strategies to enhance cryotolerance of embryos in sheep and in other species were developed mainly through the decrease of their lipid content and/or the improvement of embryo cryopreservation procedures ^[17,18]. For instance in cattle, lipolytic agents or chemicals delipidators have been successfully applied increasing cryotolerance of vitrified embryos (phenazine ethosulfate ^[19]; *trans*-10 *cis*-12 conjugated linoleic acid ^[10,20]; forskolin ^[21]). These chemicals that regulate metabolism were used to reduce embryo lipid content, inducing smaller lipid droplets and fat indexes, thus improving embryonic cryosurvival. Likewise Nagashima *et al.* ^[22] demonstrated that the high lipid content of pig embryos was responsible for their chilling sensitivity and that delipidated embryos, by centrifugation and microaspiration of polarized lipids, became more tolerant to chilling. The lipid content was also pointed as responsible for the chilling and freezing sensitivity of IVP cattle embryos and when lipid droplets were displaced by centrifugation

(mechanical delipidation) their cryosurvival was improved ^[23,24,25]. To our knowledge these techniques were not yet applied to sheep embryos.

Other approaches to improve embryo cryosurvival have been investigated. Cytoskeleton relaxant/stabilizers as cytochalasin B or D were previously used during cattle and pig embryos vitrification to prevent cellular disruption, specifically to the embryonic cytoskeleton during and after cryopreservation ^[26,27,28]. Dobrinsky *et al.* ^[29] have obtained an 82% birth rate in pig vitrified embryos using cytochalasin B. Moreover the association of two strategies, cytoskeleton relaxants and centrifugation, were successfully attempted by different authors in cattle and pig IVP embryos ^[30,31].

The cryopreservation of sheep embryos is not as widely practiced as in cattle and several methods need to be appraised ^[32]. Thus the objective of the present research was to compare different approaches for enhancing cryosurvival of sheep IVP embryos: embryo delipidation by centrifugation in the absence or presence of a cytoskeleton relaxant or by embryo culture in the presence of different doses of the *trans*-10, *cis*-12 conjugated linoleic acid isomer (CLA). The study included 3 experiments with specific objectives: in exp. 1, the effect of mechanical delipidation through centrifugation in the presence or absence of cytochalasin D on sheep embryo cryosurvival was studied; in exp. 2 we induced embryonic chemical delipidation by using different doses of CLA (25, 50 and 100 μ M) during embryo culture and their effect on embryo production and posterior cryosurvival were evaluated; in exp. 3 we compared both approaches, chemical and physical delipidation, used in previous experiments but without the groups presenting the worst results.

5.2.2 Materials and methods

All chemicals used were purchased from Sigma Aldrich Chemical Co. (Sintra, Portugal) unless specified otherwise.

5.2.2.1 Experimental design

In the present work, different approaches for enhancing cryosurvival of sheep *in vitro* produced embryos were evaluated in 3 experiments. In each experiment, embryos were produced in 8 to 9 sessions. On day 6 to day 7 (IVF=day 0), embryos were evaluated for their developmental and morphological status (grade 1, good to grade 3, poor) and then vitrified-warmed in OPS (4 sessions each). In all experiments, embryo viability after warming was evaluated by its integrity and re-expansion as well as after 3 hours of *in vitro* culture.

Experiment 1. Prior to vitrification, *in vitro* produced blastocysts of grade 1 (8 sessions) were randomly distributed into 4 groups, as follows: i) control group (n=27), embryos were placed during 20 min in holding medium (HM); ii) centrifuged group (cent; n=25), embryos were placed in HM during 10 min and then centrifuged at 15,000 X g during 10 min; iii) cytochalasin D group (cyto-D; n=24), embryos were placed in 5 $\mu\text{g mL}^{-1}$ cytochalasin D containing medium during 10 min and then transferred to HM another 10 min; iv) centrifugation+cytochalasin D group (cent+cyto-D; n=22), embryos were placed in 5 $\mu\text{g mL}^{-1}$ cytochalasin D containing medium during 10 min and then centrifuged at 15,000 X g for 10 min in HM. After warming, embryo viability was evaluated.

Experiment 2. Four experimental groups were constituted: i) control group, without supplementation; ii) CLA25 group, embryo culture media were supplemented with 25 μM CLA; iii) CLA50 group, embryo culture media were supplemented with 50 μM CLA; iv) CLA100 group, embryo culture media were supplemented with 100 μM CLA. Presumptive zygotes were randomly distributed into the culture medium treated as above (n=1538, 9 sessions). Grade 1 embryos (control n= 14, CLA25 n= 23, CLA50 n= 18) were vitrified/warmed. Due to the low number and quality of CLA100 embryos this group was excluded from the cryosurvival evaluation.

Experiment 3. Five experimental groups were assayed being their treatments as described in experiment 1 and 2 and removing the groups presenting the worst results (cent and CLA100 groups): control group, cyto-D group, cent+cyto-D group, CLA25 group and CLA50 group. As in the previous experiments, embryos production rates were evaluated (9 sessions) and grade 1 embryos vitrified/warmed (control n= 25, cyto-D, n=22, cent+cyto-D n=20, CLA25 n= 33, CLA50 n= 18).

5.2.2.2 *In vitro* embryo production

Ovine ovaries were collected at a local slaughterhouse and transported to the laboratory in Dulbecco's phosphate buffer saline (PBS, GibCo 14040-91) at 35°C. PBS was supplemented with 0.15% (w/v) of bovine serum albumin (BSA) and 0.05 mg mL^{-1} of kanamycin. At the laboratory, 2-6 mm follicles were aspirated to obtain the cumulus oocyte complexes (COC) and selected COC were matured for 22h at 38.5°C and 5% CO_2 . The maturation medium consisted of M199, 10 μM cysteamine, 0.3 mM sodium pyruvate, 10 $\mu\text{g mL}^{-1}$ FSH, 10 ng mL^{-1} EGF, 10 $\mu\text{g mL}^{-1}$ estradiol and 10 $\mu\text{L mL}^{-1}$ gentamicin ^[7].

Fresh semen from a Merino breed ram of proven fertility collected by artificial vagina was evaluated and used throughout the experiments. Collected ejaculates were kept at room temperature and light protected for up to 2 h, then washed in synthetic oviductal fluid (SOF) and centrifuged at 225 X g for 5 min.

This medium was SOF enriched with 4 mg mL⁻¹ bovine serum albumin (BSA) plus 1.5 µg mL⁻¹ glutamine and kept at the same room temperature.

Subsequently washed spermatozoa were diluted with 1 mL of fertilization medium consisting of SOF containing 20 µL mL⁻¹ BME and 10 µL mL⁻¹ MEM amino acids, 10 µL mL⁻¹ gentamicin and 10% ovine oestrus serum. Eighteen hours after insemination, presumptive zygotes were transferred into droplets of SOF supplemented with BME and MEM aminoacids, 100 µM GSH, 6 mg mL⁻¹ BSA and/or without different doses of CLA (Matreya, Pleasant Gap, Pennsylvania, USA) according to the experimental design. After assessing cleavage, embryo development proceeded until the blastocyst stage in aminoacids, 100 µM GSH and BSA supplemented SOF at the some doses as above, plus 10% fetal calf serum (SOFserum) also with/or without different doses of CLA. Embryo culture was performed at 38.5°C in a humidified atmosphere with 5% O₂, 5% CO₂ and 90% N₂. Cleavage rate was calculated as the number of cleaved embryos per number of inseminated oocytes. day 6/7 embryo developmental rate was calculated as the number of D6/7 blastocysts per number of cleaved embryos. Embryo quality was classified using a conventional morphological criteria and according to their stage of development (Grade 1: Good; Grade 2: Fair; and Grade 3: Bad) as in Romão *et al.* [7].

5.2.2.3 Blastocyst vitrification

Blastocysts were harvested on day 6 and day 7 of development and placed in holding medium (HM, M199+20% FCS) at 38.5°C. Prior to vitrification, control and CLA treated embryos were incubated 20 min in HM. Additionally accordingly to the experimental design, some embryos (2-8 per session) were placed in HM for another 10 min and centrifuged at 15000 X g in a 1.5 mL microcentrifuge tube containing HM during 10 min, while those (2-8 per session) treated with 5 µg mL⁻¹ cytochalasin D in HM were incubated for 10 min, and then for another 10 min in HM without supplementation (adapted from Tominaga *et al.* [30]). Also 2-8 embryos per session (exp. 1 and 3, group cent+cyto-D), were placed in HM plus cytochalasin D for 10 min and then centrifuged (15,000 X g) in HM for 10 min at 38.5°C.

Blastocysts vitrification was carried out by using the open pulled straw (OPS) method as described by Vajta *et al.* [33]. Briefly, embryos were first equilibrated in HM for 5 min, then in diluted (HM with 10% ethylene glycol- EG and 10% dimethyl sulfoxide-DMSO, 1 min) and concentrated (HM with 20% EG and 20% DMSO) vitrification media. Embryos were quickly placed into the superfine OPS and plunged directly into NL₂ (30 sec).

5.2.2.4 Warming and viability determination

Embryo warming was performed by placing the end of the straw directly into the HM at 38.5°C. After 2 min, blastocysts were transferred into HM for another 2 min; and then to SOFserum and cultured for 3 hours. Embryos integrity and re-expansion were assessed post-thawing and after 3 hours of *in vitro* culture as in Romão *et al.* [7].

5.2.2.5 Statistical analysis

Procedure MIXED of Statistical Analysis Systems Institute [34] was used to analyze data from embryo production (cleavage and D6/7 embryo rates) and quality. The mixed linear model included treatment as fixed effect and replicate as random effect. In addition the least square means for each treatment were calculated as well as the differences between the means and the respective t-test. Data from frozen-thawed embryos integrity were analyzed using GLIMMIX procedure from SAS. The model included treatment, embryonic developmental stage and quality as fixed effects and replicate as random effect.

Data from frozen-thawed embryos expansion (4 sessions) were analyzed using Proc Npar1way (Kruskal-Wallis test). This test was used to compare embryonic post warming expansion scored on a 1-4: without expansion = 1 point; semi-expansion = 2 points; expansion = 3 points; expansion with an excellent blastocoele = 4 points.

Results were considered statistically different when $p \leq 0.05$.

5.2.3 Results

Experiment 1

In experiment 1, oocytes (n=984) were inseminated in 8 sessions and a cleavage rate of $49.0 \pm 1.6\%$ and D6/7 embryo rate of $31.2 \pm 2.2\%$ were obtained.

After warming, significant differences ($p=0.003$) on embryo integrity were observed among groups (table 4). The integrity rate of embryos from cent group was lower than of control ($p=0.05$), cyto-D ($p=0.001$) and cent+cyto-D ($p=0.002$) groups. As represented in figure 7, the expansion rate was positively ($p<0.05$) affected by treatments. Embryos from the cent+cyto-D group presented the best results as confirmed by the highest ($p<0.05$) expansion score (figure 7, cent+cyto-D group: 2.6 ± 0.28 vs. control group: 1.8 ± 0.08 , cent group: 1.9 ± 0.20 and cyto-D group: 1.8 ± 0.31), mainly due to a higher number of expanded embryos with an excellent blastocoele. No differences in expansion rates after 3 h of culture among groups were identified.

Table 4: Effect of different protocols of lipid reduction on sheep embryo post warming evaluation (4 sessions, experiment 1).

Treatment	n	Post warming integrity (%)	Post warming expansion (%)	Expansion 3 hours post warming (%)
control	27	74.6 ± 9.2 ^a	81.5 ± 8.9	72.7 ± 9.2
centrifugation	25	50.6 ± 10.3 ^b	48.0 ± 9.2	70.8 ± 8.8
cytochalasin D	24	92.3 ± 9.7 ^a	75.0 ± 9.4	74.0 ± 9.9
centrifugation+cytochalasin D	22	90.5 ± 11.2 ^a	63.6 ± 9.8	88.9 ± 10.2

Values (least square means ± standard error) with different superscripts within individual columns are significantly different ($p < 0.05$).

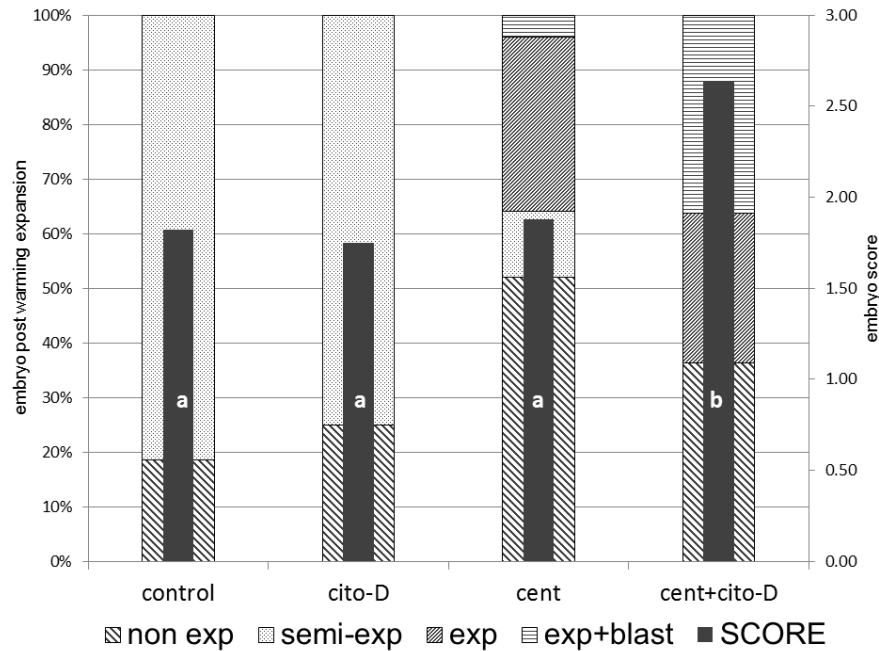


Figure 7: Evaluation of vitrified (OPS) sheep embryos after warming, previously submitted to different protocols of lipid reduction. Embryos were scored (1-4) according to the post warming expansion: without expansion (non exp) = 1, semi-expansion (semi-exp) = 2, expansion (exp) = 3 and expansion with an excellent blastocoele (exp+blast) = 4. Values with different letter within individual columns are significantly different ($p < 0.05$). Experiment 1: cytochalasin D (cyto-D, $n=24$), centrifugation (cent, $n=25$), centrifugation and cytochalasin D (cent+cyto-D, $n=22$), control (control, $n=27$) groups.

Experiment 2

Higher cleavage rates were observed in embryos cultured in the presence of 25 ($p=0.006$) and 50 μM CLA ($p=0.03$) than in control group (table 5). CLA100 presented the lowest ($p < 0.002$) D6/7 embryo production rate and quality (table 6). This group had zero grade 1 and 87.5% grade 3 embryos, lower than control ($p=0.007$), CLA25 ($p=0.03$) and CLA50 ($p=0.055$) groups (table 6).

Due to the low number and quality of CLA100 embryos this group was not included in embryo vitrification procedures. After warming, no differences were identified in embryo integrity rate among groups. However CLA supplementation during culture enhanced embryo post warming expansion (figure 8). Superior ($p < 0.02$) expansion scores were achieved in CLA groups (CLA25 group: 3.1 ± 0.29 and CLA50 group:

3.8±0.17) than in control (1.9±0.10). This latter group had no expanded blastocysts. Moreover better results were obtained in CLA50 than in CLA25 ($p<0.05$) group. No differences were identified in embryo expansion rates after 3 h of culture.

Table 5: Effect of a protocol of lipid reduction using different concentrations of *trans*-10 *cis*-12 conjugated linoleic acid isomer (CLA) during sheep embryo culture on production rates (9 sessions, experiment 2).

Treatment	Inseminated oocytes(n)	Cleavage rate (%)	D6/7 embryo rate (%)
CONTROL	575	41.8±6.1 ^a	26.1±2.6 ^a
CLA 25	346	50.9±6.2 ^b	27.7±3.2 ^a
CLA 50	356	48.9±6.2 ^b	22.2±3.2 ^a
CLA 100	261	45.8±6.4 ^{ab}	6.7±3.8 ^b

Values (least square means ± standard error) with different superscripts letters within individual columns are statistically different ($p<0.05$).

Table 6: Percentual distribution of sheep embryo morphological quality after culture with different concentrations of *trans*-10 *cis*-12 conjugated linoleic acid isomer (CLA; 9 sessions, experiment 2).

Treatment	n	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
CONTROL	70	38.6±5.7	24.3±4.7	37.1±5.9 ^a
CLA 25	55	38.2±6.4	16.4±5.3	45.5±6.6 ^a
CLA 50	45	33.3±7.1	15.6±5.9	51.1±7.3 ^{ab}
CLA 100	8	0.9±16.9	12.5±14.0	87.5±17.4 ^b

Values (least square means ± standard error) with different superscripts letters within individual columns are statistically different ($p<0.05$).

Experiment 3

As in the previous experiment, higher embryo cleavage rates were observed in embryos cultured in the presence of 25 ($p=0.004$) and 50 μ M CLA ($p=0.03$) when compared to non-supplemented group (control, table 7). No differences were identified among groups for D6/7 embryo rates or quality.

Also there were no differences among treatments in post warming integrity of embryos. As above when comparing embryo expansion scores, higher ($p<0.008$, figure 9) results were obtained after their delipidation by culture in CLA supplemented media (CLA25 group: 3.2±0.24 and CLA50 group: 3.8±0.17) or by centrifugation in the presence of cyto-D (2.8±0.29) than in control (1.9±0.07) or cyto-D alone (1.7±0.10). These latter two groups had only until semi-expanded embryos. Embryos belonging to CLA50 group had a higher expansion score ($p=0.0009$) than those from cent+cyto-D group. CLA embryos had the highest number of expanded embryos with an excellent blastocoele (figure 9). After 3 h of culture, no differences were observed in embryo expansion rates among groups.

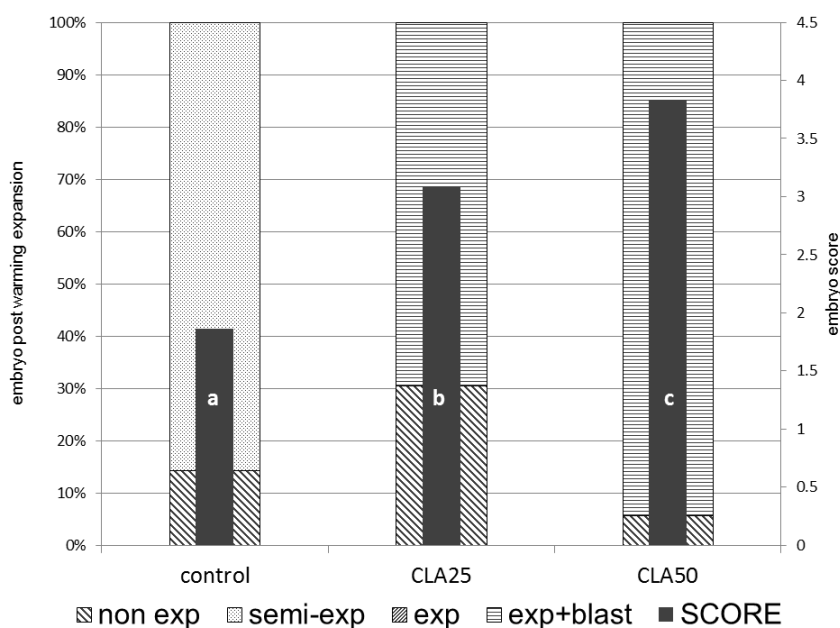


Figure 8: Evaluation of vitrified (OPS) sheep embryos after warming, previously submitted to a protocol of lipid reduction using different concentrations of *trans*-10 *cis*-12 conjugated linoleic acid isomer (CLA; 25 and 50 μ M) supplemented to the culture medium. Embryos were scored (1-4) according to the post warming expansion: without expansion (non exp) = 1, semi-expansion (semi-exp) = 2, expansion (exp) = 3 and expansion with an excellent blastocoele (exp+blast) = 4. Values with different letter within individual columns are significantly different ($p < 0.05$). Experiment 2: control (n= 14), CLA25 (n=23) and CLA50 (n=18) groups.

Table 7: Effect of different concentrations of *trans*-10 *cis*-12 conjugated linoleic acid isomer (CLA) during sheep embryo culture on production rates (9 sessions, experiment 3).

Treatment	Inseminated oocytes(n)	Cleavage rate (%)	D6/7 embryo rate (%)
Control	830	40.4 \pm 5.4 ^a	26.7 \pm 2.3
CLA25	420	49.2 \pm 5.6 ^b	30.2 \pm 3.1
CLA50	356	47.6 \pm 5.6 ^b	22.2 \pm 3.4

Values (least square means \pm standard error) with different superscripts letters within individual columns are statistically different ($p < 0.05$).

5.2.4 Discussion

Presented results show for the first time that different protocols of lipid reduction can be successfully applied to improve the cryotolerance of *in vitro* produced sheep embryos. In fact both strategies of embryo delipidation, by centrifugation in the presence of cytochalasin D or by CLA culture supplementation, enhanced the blastocyst expansion rates after vitrification/warming. Moreover of the evaluated CLA doses, 25 and 50 μ M enhanced sheep embryo production rates. Similar strategies have been previously attempted in cattle and pig embryos [10,20,30,31] but at our knowledge these techniques were herein applied for the first time in sheep embryos. Improving results in cryopreservation of IVP sheep embryos appears as a challenge that, being overcome, can spread the use of this biotechnology in this species.

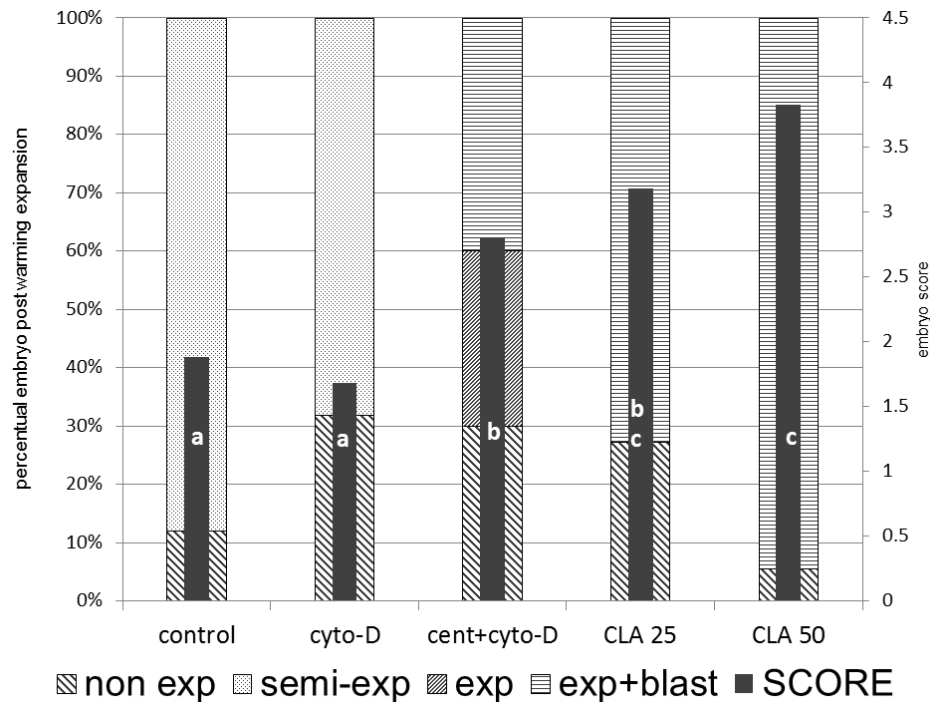


Figure 9: Evaluation of vitrified (OPS) embryos after warming, previously submitted to different protocol of lipid reduction. Embryos were scored (1-4) according to the post warming expansion: without expansion (non exp) = 1, semi-expansion (semi-exp) = 2, expansion (exp) = 3 and expansion with an excellent blastocoele (exp+blast) = 4. Values with different letter within individual columns are significantly different ($p < 0.01$). Experiment 3: control (control, $n = 25$), cytochalasin D (cyto-D, $n = 22$), centrifugation and cytochalasin D (cent+cyto-D, $n = 20$), 25 or 50 μM trans-10 cis-12 conjugated linoleic acid isomer (CLA) added in the culture medium (CLA25, $n = 33$) and (CLA50, $n = 18$) groups.

In sheep there are several constraints already identified for *in vitro* embryo production and cryotolerance. As in other species, the excessive abundance of intracellular lipid droplets in these embryos has been correlated with their impaired quality and cryoresistance [8,14,10]. Several ways of reducing lipid content in embryos of different species, either using physical techniques and/or chemical delipidating agents improving post-thawed viability have been reported [23,24,30,19,11]. As referred the centrifugation of IVP embryos prior to freezing induces the polarization of lipid droplets. These polarized lipid droplets could be further aspirated by micromanipulation [22] but observations of Ushijima *et al.* [24] and Li *et al.* [31] demonstrated that the displacement of lipid droplets by centrifugation only, was also effective in improving cattle and pig embryos cryosurvival. Embryo centrifugation has the advantage of reducing labour and disease transmission associated with the invasive micromanipulation technique [31,35]. However in the present study, sheep embryos submitted to centrifugation prior to vitrification had the lowest (cent group, $p = 0.003$) post warming integrity rate; Somfai *et al.* [36] had also reported a detrimental effect of centrifugation applied before vitrification in pig oocytes and embryos viability. Conversely the association of centrifugation and the

presence of cytochalasin D has overcome this negative effect, confirming our preliminary results^[37] of a possible role of cytochalasin D in protecting mechanical damage of centrifuged embryos during cryopreservation. Moreover, embryos from the cent+cyto-D group showed the highest ($p<0.05$) expansion score mainly due to an enhanced number of expanded embryos with an excellent blastocoele (figure 7).

Cytoskeleton stabilizers as cytochalasins have been advocated as a useful tool for preserving embryo integrity during the freezing process. In fact, if the cytoskeleton is damaged or de-stabilized it can lead to irreparable cell destruction. These blastomeres destruction is closely related to embryo quality. Better quality embryos (grade 1 and 2) presented small cytoskeleton damage after thawing, while cryopreserved grade 3 embryos showed a high level of cytoskeleton disorganization^[32,38]. Cytochalasin induces microfilament depolymerization prior to and during vitrification^[39], acting as microfilaments inhibitor thus preventing actin polymerization and making the plasmatic membrane and cytoskeleton more elastic. So embryo microfilaments and cytoskeletal architecture are not destroyed during micromanipulation, but also become more resistant to the osmotic stress induced by the exposure and removal of cryoprotectants during the vitrification/warming process^[26,28]. Currently two cytochalasins, B and D, are being tested to improve the survival of oocytes and embryos to cryopreservation. Ushijima *et al.*^[24] reported that treatment with cytochalasin B and centrifugation without micromanipulation did not improve the cryotolerance of bovine embryos but Tominaga *et al.*^[30] and Franco and Hansen^[28] showed an increase in the cryotolerance of cattle 16-cell embryos and blastocysts treated with cytochalasin D or cytochalasin B, respectively. Dobrinsky *et al.*^[39] have also successfully used cytochalasin B in pig embryos. However in the present study, the survival rates of sheep embryos after warming were not superior to control when using cytochalasin D alone. As far as we know this technique has never been previously applied to sheep embryos although cytoskeleton stabilizers were used in sheep oocyte vitrification without promising results in some studies^[40,41] and positive effects in others^[42]. As referred herein better results were obtained using cytochalasin D and centrifugation together. Nonetheless superior post warming expansion rates were obtained in CLA50 embryos (experiment 3).

Regarding *in vitro* embryo production, in the present study a cleavage rate ranging from 49.0% (exp. 1) to 40.4% (exp. 3) in control groups were obtained. These results are similar to those of Romão *et al.*^[7] but lower than some others reports^[2,43]. Moreover the reported blastocyst rate of 31.2% (exp. 1), to 26.1% (exp. 2), are in the range of previous results. When CLA was supplemented to embryo culture medium, the cleavage rate was enhanced ($p<0.05$) either with 25 or 50 μ M CLA, in both experiment 2 and 3, although

there was no significant differences in blastocyst rates at these doses (table 5 and 7). Conjugated linoleic acid is one of the isomers of linoleic acid; specifically the *trans*-10 *cis*-12 linoleic acid isomer (CLA) is of primary physiological importance with many promising beneficial effects, namely anti-mutagenic, antioxidant and anti-carcinogenic [44,45]. In IVP embryo production it has been demonstrated that this polyunsaturated fatty acid (PUFA) reduces lipid accumulation during culture [10,20,46] improving embryo cryopreservation tolerance and survival [10,20,47]. The use of CLA in the culture media can reduce the intake and synthesis of fatty acids in adipocytes [44], preventing also the excessive fatty acid deposition in embryos. In fact in CLA presence, IVP cattle embryos show smaller lipid droplets and fat embryo indexes [10,20]. It has also been demonstrated the interference of CLA on lipid metabolism during maturation and a positive effect on bovine oocyte developmental competence [48].

In the present study we tested three different concentrations of CLA (25, 50 and 100 μ M) in the culture medium because there were no previous references for sheep embryos. Although in cattle the above cited results have been obtained with a dose of 100 μ M CLA, presented results showed that this concentration is almost lethal for sheep embryo production leading to lower blastocyst rate ($p < 0.05$, experiment 2, table 5) and poor quality embryos ($p < 0.05$, table 6). Stinshoff *et al.* [49] also reported a decrease in cattle blastocysts developmental rate in CLA groups either with 50 or 100 μ M doses supplementation. However in the present study sheep embryos supplemented with 50 μ M of CLA during *in vitro* culture showed an improved cleavage rate and cryosurvival. Despite these encouraging results, there were no differences in blastocyst expansion after 3 hours of culture as also occurred in exp 1 and 2. As herein Green *et al.* [50] reported that, in sheep vitrified embryos, increasing the interval between warming and transfer has a detrimental effect on subsequent embryo viability. They concluded that better results are obtained with embryo direct transfer instead of embryo warming and culture before transfer.

Regarding CLA embryo supplementation, the discrepancies among studies may be related to species specificities and/or to the use of different culture systems. Herein, besides CLA, sheep embryos were cultured in SOF medium with amino acids, BSA, 10% serum and 100 μ M GSH. In Pereira *et al.* [10,20], cattle embryos were cultured on a granulosa cell monolayer under mineral oil, in TCM199, 10% serum, 100 μ M GSH and 100 μ M CLA, while Stinshoff *et al.* [49] employed an oil-free culture system using SOF medium with amino acids, BSA and 50 or 100 μ M CLA. It is possible that in the absence of a free radical scavenger, this PUFA supplementation to embryo culture media may exert a detrimental effect as reported by Reis *et al.* [51] and Khalil *et al.* [52]. Moreover these authors stated that regardless the endogenous anti-oxidant status within

the embryo, an additional need to prevent the oxidation of any fatty acid added to the culture medium prior to their assimilation into the embryo is of primordial importance ^[51]. Once inside the embryo, CLA as other PUFAs may follow the mitochondrial β -oxidation to produce energy or to synthesize fatty acids to be used during development ^[18,51,53]. These physiological complex processes within the mitochondria leads to the generation of reactive oxygen species (ROS) that must be neutralized ^[19,53]. Oxidative stress reflects an imbalance between production of ROS and cellular antioxidant defense mechanisms ^[52,53]. Preimplantation embryos are particularly sensible to ROS ^[53]. Thus special attention should be given to provide constituents within the culture medium, such as nutrients and anti-oxidants, to minimize the oxidative stress of IVP embryos supplemented with PUFA. On the other hand, as referred, CLA is a biologically active compound capable of interfering in lipid metabolism reducing the fatty acids up-take, their synthesis and also increasing lipolysis through the regulation of adipogenic genes, the activation of the peroxisome proliferator-activated receptors (PPARs), cytosolic perilipins and/or key enzymes of lipid metabolism ^[18,44,49,54]. The exact mechanism through which this isomer influences embryo lipid metabolism remains elusive. The mechanism of CLA action on *in vitro* embryo produced physiology and resistance to the cryopreservation process demands to be further enlightened.

In conclusion, excessive lipid deposition and ROS generation seem to be two major factors hampering *in vitro* embryo production and cryosurvival. The results of the present study showed that different protocols of lipid reduction applied to IVP sheep embryos improved cryotolerance. Our data indicate a positive effect of 25 or 50 μ M CLA plus GSH supplementation on the developmental competence of sheep IVP embryos as reflected by improved cleavage and post warming expansion rates. Additionally IVP embryos centrifugation in the presence of cytochalasin D prior to vitrification also improved post warming survival. Further studies investigating if these strategies could be applied together improving sheep embryo resistance to cryopreservation and pregnancy rate after transfer should be addressed.

5.2.5 References

- [1]Alabart, JL, Lahoz, B, Folch, J, Sánchez, P, Echegoyen, E, Cocero, MJ. Effect of a defined or undefined *in vitro* maturation medium on cleavage rate and embryo development in sheep. 26th Annual Meeting AETE, Kuopio, Finland, 2000.
- [2]Dattena M, Ptak G, Loi P, Cappai P. Survival and viability of vitrified *in vitro* and *in vivo* produced ovine blastocysts. *Theriogenology* 2000;53:1511-1519.
- [3]Accardo C, Dattena M, Pilichi S, Mara L, Chessa B, Cappai P. Effect of recombinant human FSH and LH on *in vitro* maturation of sheep oocytes; embryo development and viability. *Anim Reprod Sci* 2004;81:77-86.

- [4]Cocero MJ, Alabart JL, Hammami S, Martí JI, Lahoz B, Sánchez P, Echegoyen E, Beckers JF, Folch J. The efficiency of *in vitro* ovine embryo production using an undefined or a defined maturation medium is determined by the source of the oocyte. *Reprod Dom Anim* 2011;46:463-470.
- [5]Gibbons A, Cueto MI, Pereyra Bonnet F. A simple vitrification technique for sheep and goat embryo cryopreservation. *Small Rumin Res* 2011;95:61-64.
- [6]Dieleman SJ, Hendriksen PJM, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TAM, Niemann H, Gadella BM, Bevers MM, Vos PLAM. Effect of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology* 2002;57:5-20.
- [7]Romão R, Marques CC, Baptista MC, Vasques MI, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Plancha C, Rodrigues P, Pereira RM. Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen. *Small Rumin Res* 2013;110:36-41.
- [8]Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos. *Biol Reprod* 1995;53:1385-1391.
- [9]Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol.Reprod Dev* 2002;61:234-248.
- [10]Pereira RM, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Chagas e Silva J, Silva Pereira M, Marques CC. Cryo-survival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t, 12c CLA). *Anim. Reprod. Sci* 2007;98:293-301.
- [11]Pereira RM, Marques CC. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank* 2008;9:267-277.
- [12]Abe H, Hoshi H. Evaluation of bovine embryos produced in high performance serum-free media. *J Reprod Dev* 2003;49(3):193-202.
- [13]Thompson JG. *In vitro* culture and embryo metabolism of cattle and sheep embryos—a decade of achievement. *Anim Reprod Sci* 2000;30:273–280.
- [14]Abe H, Shiku H, Aoyagi S, Hoshi H. *In vitro* culture and evaluation of embryos for production of high quality bovine embryos. *J Mamm Ova Res* 2004;2:22-30.
- [15]Lapa M, Marques CC, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Pereira RM. Fatty acid profile of oocytes and blastocysts during *in vitro* bovine embryo production. *Reprod Dom Anim* 2005;40(4):365.
- [16]Rooke JA, McEvoy TG, Ashworth CJ, Robinson JJ, Wilmut I, Young LE, Sinclair KD. Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology* 2007;67:639-647.
- [17]Arav A. Cryopreservation of oocytes and embryos. *Theriogenology* 2014;8:96-102.
- [18]Prates EG, Nunes JT, Pereira RM. A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production. *Mediators Inflamm* 2014;Article ID 692067:1-11.
- [19]Barceló-Fimbres M, Seidel Jr GE. Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during *in vitro* culture of bovine embryos on embryonic development after cryopreservation. *Mol Reprod Dev* 2007;74:1395-1405.
- [20]Pereira RM, Carvalhais I, Pimenta J, Baptista MC, Vasques MI, Horta AE, Santos IC, Marques MR, Reis A, Pereira MS, Marques CC. Biopsied and vitrified bovine embryos viability is improved by *trans*10, *cis*12 conjugated linoleic acid supplementation during *in vitro* embryo culture. *Anim Reprod Sci*, 2008;106(3-4):322-32.
- [21]Sanches BV, Marinho LSR, Filho BDO, Pontes JHF, Basso AC Meirinhos MLG, Silva-Santos KC, Ferreira CR, Seneda MM. Cryosurvival and pregnancy rates after exposure of IVF-derived *Bos indicus* embryos to forskolin before vitrification. *Theriogenology* 2013;80:372-377.
- [22]Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG, Seamark RF, Nottle MB. Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. *Biol Reprod* 1994, 51:618-622.

PAPERS SUBMITTED TO PUBLICATION

- [23]Leibo SP, Pollard JW, Martino A. Chilling and freezing sensitivity of “reassembled” *in vitro*-derived bovine embryos. *Theriogenology* 1995;43(1):265[abstract].
- [24]Ushijima H, Yamakawa H, Nagashima H. Cryopreservation of bovine pre-morula-stage *in vitro* matured/*in vitro* fertilized embryos after delipidation and before use in nucleus transfer. *Biol Reprod* 1999;60:534-539.
- [25]Diez, C, Heyman, Y, Le Bourhis, D, Guyader-Joly, C, Degrouard, J, Renard, JP. Delipidating *in vitro*-produced bovine zygotes: effect on further development and consequences for freezability. *Theriogenology* 2001;55:923-936.
- [26]Dobrinisky JR, Pursel VG, Long CR, Johnson LA. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. *Biol Reprod* 2000;62:564-570.
- [27]Dobrinisky JR. Advancements in cryopreservation of domestic animal embryos. *Theriogenology* 2002;57:285-302.
- [28]Franco M, Hansen PJ. Effects of hyaluronic acid in culture and cytochalasin B treatment before freezing on survival of cryopreserved bovine embryos produced *in vitro*. *In Vitro Cell. Dev Biol – Animal* 2006;42:40-44.
- [29]Dobrinisky JR, Nagashima H, Purse VG, Schreier LL, Johnson LA. Cryopreservation of morula and early blastocyst stage swine embryos: birth of litters after embryo transfers. *Theriogenology* 2001;55:303 [abstract].
- [30]Tominaga K, Shimizu M, Ooyama S, Isaïke Y. Effect of lipid polarization by centrifugation at different developmental stages on post-thaw survival of bovine *in vitro* produced 16-cell embryos. *Theriogenology* 2000;53:1669-1680.
- [31]Li R, Murphy CN, Spate L, Wax D, Isom C, Rieke A, Walters EM, Samuel M, Prather RS. Production of piglets after cryopreservation of embryos using a centrifugation-based method for delipidation without micromanipulation. *Biol Reprod* 2009, 80:563-571.
- [32]Dalcin L, Silva RC, Paulini F, Silva BD, Neves JP, Lucci CM. Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos. *Cryobiology* 2013;67(2):137-45.
- [33]Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open pulled straws (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev* 1998;51:53-58.
- [34]SAS Institute. SAS® 9.1.2 for Microsoft Windows. SAS International, Heidelberg, Germany. 2004.
- [35]Spate LD, Murphy CN, Prather RS. High-throughput cryopreservation of *in vivo*-derived swine embryos. *PLOS ONE* 2013;8(6):1-5. doi:10.1371/journal.pone.0065545
- [36]Somfai T, Kashiwazaki N, Ozawa M, Nakai M, Maedomari N, Noguchi J, Kaneko H, Nagai T, Kikuchi K. Effect of centrifugation treatment before vitrification on the viability of porcine mature oocytes and zygotes produced *in vitro*. *J Reprod Dev* 2008;54(3):149-155.
- [37]Romão R, Marques C, Baptista M, Barbas J, Horta A, Carolino N, Bettencourt E, Pereira R. Cryopreservation of ovine *in vitro* produced embryos using centrifugation and cytochalasin D. *Reprod Dom Anim* 2013;48(Suppl.1):104.
- [38]Dobrinisky JR. Cellular approach to cryopreservation of embryos. *Theriogenology* 1996;45:17-26.
- [39]Dobrinisky JR, Long CR Johnson LA. Stability of microfilaments during swine embryo cryopreservation. *Theriogenology* 1997;47(1):343[abstract].
- [40]Silvestre MA, Yáñez J, Salvador I, Santolaria P, López-Gatius F. Vitrification of pre-pubertal ovine cumulus-oocyte complexes: effect of cytochalasin B pre-treatment. *Anim Reprod Sci* 2006;93:176-182.
- [41]Bogliolo L, Ariu F, Fois S, Rosati I, Zedda MT, Leoni G, Succu S, Pau S, Ledda S. Morphological and biochemical analysis of immature ovine oocytes vitrified with or without cumulus cells. *Theriogenology* 2007;68:1138-1149.
- [42]Zhang, J, Nedambale, TL, Yang M, Li J. Improved development of ovine matured oocyte following solid surface vitrification (SSV): effect of cumulus cells and cytoskeleton stabilizer. *Anim Reprod Sci* 2009;110:46-55.
- [43]Pereira RM, Mesquita P, Batista M, Baptista MC, Barbas JP, Pimenta J, Santos IC, Marques MR, Vasques MI, Silva Pereira M, Santos Silva F, Oliveira Sousa MC, Fontes CMG, Horta AEM, Prates JAM, Marques CC. Doppel gene polymorphisms in Portuguese sheep breeds: Insights on ram fertility. *Anim Reprod Sci* 2009;114:157-166.
- [44]Pariza MW, Park Y, Cook ME. The biologically active isomers of conjugated linoleic acid. *Prog Lipid Res* 2001;40:283-298.

- [45]Crumb DJ. Conjugated linoleic acid (CLA) – an overview. *International Journal of Applied Research in Natural Products* 2011;4(3):12-18.
- [46]Pereira, RM, Baptista, MC, Vasques, MI, Horta, AEM, Porta PV, Bessa RJB, Marques, CC. Post-thawing resistance of bovine embryos is improved by *trans*-10, *cis*-12conjugated linoleic acid (CLA). In: *Proceedings of 15th International Congress on Animal Reproduction, Porto Seguro, Brasil, 2004.*
- [47]Accorsi, MF. Assessment of bovine embryos grown *in vitro* in the presence of fatty acids and their survival after cryopreservation. Dissertation – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, Brasil, 2008.
- [48]Lapa M, Marques CC, Alves SP, Vasques MI, Baptista MC, Carvalhais I, Silva Pereira M, Horta AE, Bessa RJ, Pereira RM. Effect of *trans*-10 *cis*-12 conjugated linoleic acid on bovine oocyte competence and fatty acid Composition. *Reprod Dom Anim* 2011;46:904-910.
- [49]Stinshoff H, Wilkening S, Hanstedt A, Bollwein H, Wrenzycki C. Dimethylsulfoxide and conjugated linoleic acids affect bovine embryo development *in vitro*. *Reprod Fertil Dev* 2014;26(4):502-10.
- [50]Green RE, Santos BFS, Sicherle CC, Landim-Alvarenga FC, Bicudo SD. Viability of OPS vitrified sheep embryos after direct transfer. *Reprod Dom Anim* 2009;44:406-410.
- [51]Reis, A, Rooke JA, McCallum GJ, Staines ME, Ewen M, Lomax MA, McEvoy, TG. Consequences of exposure to serum, with or without vitamin E supplementation, in terms of the fatty acid content and viability of bovine blastocysts produced *in vitro*. *Reprod Fertil Dev* 2003;15:275-284.
- [52]Khalil WA, Marei WFA, Khalid M. Protective effects of antioxidants on linoleic acid–treated bovine oocytes during maturation and subsequent embryo development. *Theriogenology* 2013;80:161-168.
- [53]Harvey AJ, Kind KL, Thompson JG. REDOX regulation of early embryo development. *Reproduction* 2002;123:479-486.
- [54]Granlund, L, Pedersen, JI, Nebb, HI. Impaired lipid accumulation by *trans*10, *cis*12 CLA during adipocyte differentiation is dependent on timing and length of treatment. *Biochim Biophys Acta* 2005;1687:11-22.

5.3 Ultrastructure of *in vitro* produced sheep embryos: effect of *trans*-10 *cis*-12 conjugated linoleic acid (CLA) in fresh and cryopreserved blastocysts

Romão, R.^{1,2}, Marques, C.C.¹, Bettencourt, E.², Baptista, M.C.¹, Barbas, J.P.^{2,3}, Carolino, N.^{1,3,4},
Oliveira, E.⁵, Sousa, M.⁵, Pereira, R.M.L.N.^{1,3,4}

¹Unidade de Biotecnologia e Recursos Genéticos, Instituto Nacional de Investigação Agrária e Veterinária (INIAV)-Santarém, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal

²Escola de Ciências e Tecnologia, “ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas” - Universidade de Évora – Núcleo da Mitra, Ap. 94, 7002- Évora, Portugal

³CIISA, Universidade de Lisboa, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal

⁴Escola Universitária Vasco da Gama, Av. José R. Sousa Fernandes, Campus Universitário – Bloco B, Lordemão, 3020-210 Coimbra, Portugal

⁵Department of Microscopy, Laboratory of Cell Biology, Multidisciplinary Unit for Biomedical Research-UMIB, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, 4050-313 Porto, Portugal

Abstract

Preservation of *in vitro* produced (IVP) sheep embryos is required for the full application of this technology in the future. Nonetheless, IVP embryos are difficult to cryopreserve in this species. Cryopreserved sheep embryos show ultrastructural changes that may reduce their viability after freezing/warming. Their high lipid content is often accused of being one of the possible causes of this failure. Attempts have been made to reduce lipid content of embryos, and *trans*-10, *cis*-12 octadecadienoic acid (CLA) has been successfully used in bovine. In the present study we evaluate the ultrastructural changes in sheep embryos cultured in the presence of CLA prior to and after vitrification. IVP zygotes were allocated either in a group where 25 μ M CLA was added (CLA25, n=346) to the culture medium or in a non-supplemented group (control, n=497). Grade 1 and 2 blastocysts were vitrified by OPS. For ultrastructural evaluation, four groups were formed and processed by electron microscopy: control fresh (CF, n=3), control vitrified (CV, n=3), CLA fresh (CLA25F, n=3) and CLA vitrified (CLA25V, n=3). Higher cleavage ($43.2\pm 5.3\%$ vs. $36.4\pm 5.1\%$, $p=0.04$) and D6/7 embryo rates ($34.4\pm 4.4\%$ vs. $20.2\pm 4.6\%$, $p=0.009$) were observed in embryos cultured in the presence of CLA. The quality of produced embryos was superior in CLA25 group, with more grade 1 ($p=0.004$) and less grade 2 ($p=0.03$) embryos. In post warming evaluation, CLA25 embryos showed higher integrity than those of control ($93.3\pm 10.0\%$ vs. $66.7\pm 10.0\%$, $p=0.04$), although no differences ($p>0.05$) were observed in post warming expansion rate. Ultrastructure of fresh embryos (CF, CLA25F) revealed abundant mitochondria, associated to smooth endoplasmic reticulum, but the number of Golgi complexes and membrane preservation seems higher in CLA25F, where peripheral cells presented more microvilli and less cellular debris in the perivitelline space. Cytoplasmic dense vesicles were more abundant in CLA25F while light vesicles and lipid droplets were less abundant when compared to CF. Embryos from CLA25V presented also more abundant mitochondria showing transverse cristae compared to CV, where severe signs of cell lysis were seen with disrupted membranes and the presence of more cell debris. In conclusion, these results demonstrate that it is possible to increase the production rates and quality of IVP sheep embryos using CLA in the culture medium. At the ultrastructural level, this supplementation allowed a higher preservation of cell structures and reduction of lysis, phenomena that usually are associated to the cryopreservation of sheep embryos. These positive effects improved sheep embryo cryotolerance.

Keywords: sheep; embryo; cryopreservation; ultrastructure; CLA

5.3.1 Introduction

The *in vitro* production of sheep embryos is an expensive and demanding procedure. However, for commercial and research purposes, *in vitro* embryo production can be of interest due to the possibility of slaughterhouse collection of oocytes and use of high quality sperm, either fresh or cryopreserved. Moreover the cryopreservation of *in vitro* produced (IVP) embryos is indispensable for the application of this biotechnology in the future. However, although some advances have been achieved in sheep embryo cryopreservation in the last years, results are not yet sufficient to allow its current use (Thibier and Guérin, 2000; Romão *et al.*, 2014). Therefore an effort to raise sheep IVP embryo production and cryosurvival rates is necessary to enable its practical application.

The methodologies of IVP embryos try to mimic natural embryo development and it is known that culture media composition can influence their viability (Pereira and Marques, 2008). Serum is frequently used in culture media as a free radical scavenger providing also an important nutritive role (Thompson, 2000) but serum-containing media was associated to an increase in lipid content of IVP embryos (Thompson *et al.* 1995; Abe and Hoshi, 2003; Pereira *et al.*, 2007). This higher lipid content has been associated to an increased sensitivity to chilling and related to ultrastructural changes (Crosier *et al.*, 2000; Pereira *et al.*, 2007; Dalcin *et al.*, 2013). In fact, IVP embryos are usually more susceptible to freezing when compared to their *in vivo* counterparts (Pollard and Leibo, 1993; Hasler *et al.*, 1995; Fair *et al.*, 2001; Dattena *et al.*, 2004). It is then important to find mechanisms for decreasing cryosensitivity in order to spread the use of this source of embryos.

Previous studies were able to improve the resistance to cryopreservation of pig and cattle IVP embryos by adding lipolytic agents or chemicals delipidators to culture media (Men *et al.*, 2006; Barceló-Fimbres and Seidel Jr, 2007; Pereira *et al.*, 2007, 2008). Namely the *trans*-10, *cis*-12 octadecadienoic acid (CLA) has been used during culture of cattle IVP embryos in serum-containing medium significantly improving their cryopreservation survival rates (Pereira *et al.*, 2007, 2008). This CLA isomer was capable of reducing embryo lipid accumulation during *in vitro* culture. Others have reported changes in the fatty acid composition and lipids droplets' size and location within the cytoplasm during oocyte maturation in CLA supplemented media (Prates *et al.*, 2013a, b). Also the anti-carcinogenic properties, anti-atherogenic and anti-obesity effects of CLA have been extensively reported (Pariza *et al.*, 2001; Prates *et al.*, 2014). Thus this strategy of supplementing CLA to culture media could be applied to reduce the lipid content of sheep IVP embryos improving their cryoresistance. Moreover different degrees of cellular damage were observed in the

ultrastructure of *in vivo* produced sheep embryos after cryopreservation associated to different fertility rates after transfer (Bettencourt *et al.*, 2009). So the ultrastructural evaluation of these IVP sheep embryos could be useful in order to identify the main changes associated to cryopreservation and their relations to expected survival rates.

The objective of the present work was to evaluate the effect of CLA chemical delipidation during *in vitro* culture of sheep embryos, on its production rates and cryosurvival as well as on embryo morphology and ultrastructure prior to and after thawing.

5.3.2 Materials and methods

All chemicals used were purchased from Sigma Aldrich Chemical Co. (St. Louis, USA) unless specified otherwise. Manipulation of animals was conducted at the experimental farm of INIAV in compliance with the requirements of the European Union for farm animal welfare and the Portuguese authority guidelines for animal experimentation.

5.3.2.1 Experimental design

The effect of supplementing 25 μ M of *trans*-10, *cis*-12 octadecadienoic acid (CLA, Matreya, Pleasant Gap, Pennsylvania, USA; Romão *et al.*, 2014) during culture on embryo production and posterior cryosurvival was investigated in 6 sessions. Therefore presumptive zygotes were randomly distributed into 2 groups: CLA25 group (n=346), embryo culture media was supplemented with 25 μ M CLA and control group (n=497), without supplementation. Embryo production rates and morphological quality at day 6 and 7 were evaluated. Grade 1 and 2 embryos were vitrified by OPS and post-warming embryo integrity and re-expansion rates were calculated.

To study the effect of CLA in fresh and cryopreserved embryos at the ultrastructural level, using electron microscopy, four experimental groups were constituted: control group of fresh embryos (CF, n=3) and control group of vitrified/warmed embryos (CV, n=3), both with embryos cultured without supplementation; CLA25F group (n=3), fresh produced embryos cultured in media supplemented with 25 μ M CLA; and CLA25V group (n=3), vitrified/warmed embryos produced in culture media supplemented with 25 μ M CLA.

5.3.2.2 *In vitro* embryo production

Ovine ovaries were collected at a local slaughterhouse and transported to the laboratory in Dulbecco's phosphate buffer saline (PBS, GibCo 14040-91) at 35°C. PBS was supplemented with 0.15% (w/v) of bovine

serum albumin (BSA) and 0.05 mg mL⁻¹ of kanamycin. At the laboratory, 2-6 mm follicles were aspirated to obtain the cumulus oocyte complexes (COC) and selected COC were matured for 22h at 38.5°C and 5% CO₂. The maturation medium consisted of M199, 10 µM cysteamine, 0.3 mM sodium pyruvate, 10 µg mL⁻¹ FSH, 10 ng mL⁻¹ EGF, 10 µg mL⁻¹ estradiol and 4 µL mL⁻¹ gentamicin (Romão *et al.*, 2013).

Fresh semen from a Merino breed ram of proven fertility was used throughout the experiments. Collected ejaculates were kept at room temperature and light protected for up to 2 h, then washed in synthetic oviductal fluid (SOF) and centrifuged at 225 X g for 5 min. This medium was SOF enriched with 4 mg mL⁻¹ BSA plus 1.5 µg mL⁻¹ glutamine and kept at the same room temperature. Subsequently washed spermatozoa were diluted with 1 mL of fertilization medium consisting of SOF containing 1.5 µg mL⁻¹ glutamine, 20 µL mL⁻¹ BME and 10 µL mL⁻¹ MEM amino acids, 4 µL mL⁻¹ gentamicin and 10% ovine oestrus serum. Eighteen hours after insemination, presumptive zygotes were transferred into droplets of SOF supplemented with BME and MEM aminoacids, 6 mg mL⁻¹ BSA, 100 µM GSH and/or without 25 µM CLA. After assessing cleavage, embryo development proceeded until the blastocyst stage in aminoacids, GSH and BSA supplemented SOF at the same doses as above, plus 10% fetal calf serum (SOFserum) also with/or without CLA. Embryo culture was performed at 38.5 °C in a humidified atmosphere with 5% O₂, 5% CO₂ and 90% N₂.

5.3.2.3 Embryo evaluation

Embryo production rates were evaluated at day 2 (cleavage) and day 6/7 (morulae and blastocysts). Cleavage rate was calculated as the number of cleaved embryos per number of inseminated oocytes. Day 6/7 embryo developmental rate was calculated as the number of D6/7 morulae and blastocysts per number of cleaved embryos.

Morphological quality scoring of day 6 and 7 embryos was performed based on morphological criteria and developmental stage in accordance to the International Embryo Transfer Society guidelines (Stringfellow and Seidel, 1998). Embryos were scored as grade 1 (excellent or good) when presenting a symmetrical and spherical cell mass with ≥75% of intact cells (<25% of degenerated cells/cell fragments), with individual cells showing an uniform size, color and density and grade 2 embryos (fair) had a cell mass with ≥50% of intact cells (25-50% of degenerated cells/cell fragments), with individual cells presenting moderate irregularities in size, color and density.

5.3.2.4 Blastocyst vitrification

Blastocysts vitrification was carried out by using the open pulled straw (OPS) method as described by Vajta *et al.* (1998). Briefly, day 6 and 7 embryos were first equilibrated in holding medium (HM, M199+20% FCS) for 5 min, then in diluted (HM with 10% ethylene glycol- EG and 10% dimethyl sulfoxide-DMSO, 1 min) and concentrated (HM with 20% EG and 20% DMSO) vitrification media. Embryos were quickly placed into the superfine OPS and plunged directly into NL_2 (30 sec).

5.3.2.5 Warming and viability determination

Embryo warming was performed by placing the end of the straw directly into the HM at 38.5°C. After 2 min, blastocysts were transferred into HM for another 2 min; and then to SOFserum and cultured for 1 hour. Embryos integrity and re-expansion were assessed as in Romão *et al.* (2013).

5.3.2.6 Electron microscopy processing

Fresh grade 1 blastocysts of CLA25F (n=3) and CF (n= 3) groups were processed for electron microscopy evaluation. Vitrified/warmed embryos of groups CV (n=3) and CLA25V (n=3) were also processed one hour after thawing. Embryos were fixed in Karnovsky (2 h, 4°C), washed with 0.15 M sodium cacodylate buffer, pH 7.3 (overnight, 4°C), post-fixed (1 h, 4°C) in 1% OsO_4 in buffer containing 0.8% hexanocyanoferrate potassium [$K_3Fe^{3+}(CN)_6$], and washed for 15 min in buffer. They were then dehydrated through a graded series of ethanol (50, 70, 90, 2x100%; 30 min each, at room temperature), followed by propylene oxide (15 min), impregnated with propylene oxide:epon (3:1, 1 h; 1:1, at room temperature, overnight, 4°C; 1:3, 4 h at room temperature) and embedded in Epon (7 h at room temperature, 3 days at 60°C). Sections were cut in a Leica ultramicrotome with a Diatome knife. Semithin sections were stained with aqueous azur II:methylene blue (1:1). Ultrathin sections were collected on 200 mesh copper grids (Taab) and stained with 3% aqueous uranyl acetate (20 min) and Reynolds lead citrate (10 min). Sections were observed in a transmission electron microscope JEOL 100CXII operated at 60kV.

5.3.2.7 Statistical analysis

Procedure MIXED of Statistical Analysis Systems Institute (SAS Inst., 2004) was used to analyze data from embryo production (cleavage and D6/7 embryo rates) and quality. The mixed linear model included treatment as fixed effect and replicate as random effect. In addition, the least square means for each treatment were calculated as well as the differences between the means and the respective t-test. Data from

frozen-thawed embryos integrity were analyzed using GLIMMIX procedure from SAS. The model included treatment, embryonic developmental stage and quality as fixed effects and replicate as random effect. Results were considered statistically different when $p \leq 0.05$.

5.3.3 Results

As described in table 8, higher cleavage and D6/7 embryo rates were observed in embryos cultured in the presence of 25 μM CLA (CLA25 group) compared to control group ($p=0.04$ and $p=0.009$, respectively). The quality of produced embryos was also superior in CLA25 group, presenting a higher proportion of grade 1 ($p=0.004$) and a lower proportion of grade 2 ($p=0.03$) embryos when compared to control group (table 9).

After warming, CLA25 group cryopreserved embryos showed higher integrity rates than those of control group ($p=0.04$), despite no significant ($p>0.05$) differences in the post warming expansion rate (table 10) were observed.

Table 8: Production rates (6 sessions) of *in vitro* produced sheep embryos cultured with 25 μM of *trans*-10 *cis*-12 linoleic acid isomer (CLA25 group) or without (control group).

treatment	n	cleavage (%)	D6/7 embryo (%)
CONTROL	497	36.4 \pm 5.1 ^a	20.2 \pm 4.6 ^a
CLA25	346	43.2 \pm 5.3 ^b	34.4 \pm 4.4 ^b

Data (least square means \pm standard error) within the same columns with different superscripts letters are statistically different ($p \leq 0.05$).

Table 9: Percentual distribution of morphological quality (6 sessions) of *in vitro* produced sheep embryos cultured with 25 μM of *trans*-10 *cis*-12 linoleic acid isomer (CLA25 group) or without (control group).

treatment	n	grade 1 (%)	grade 2 (%)	grade 3 (%)
CONTROL	38	23.7 \pm 7.6 ^a	29.0 \pm 6.2 ^a	47.4 \pm 8.0
CLA25	42	54.8 \pm 7.3 ^b	9.5 \pm 5.9 ^b	35.7 \pm 7.6

Data (least square means \pm standard error) within the same columns with different superscripts letters are statistically different ($p \leq 0.05$).

At ultrastructural level CLA25F embryos (fig. 10) presented well preserved cells, with well-defined nucleus showing a euchromatic nucleoplasm and one or more reticular nucleolus, associated to high transcription activity. Abundant mitochondria, associated to smooth endoplasmic reticulum, and Golgi complexes were observed in both CLA25F and CF embryos. Cisternae of endoplasmic reticulum were also associated to nuclear membrane (fig. 10F). Membrane appeared to be better preserved at CLA25F embryos

when compared to CF counterparts, in which signs of disrupted nuclear membrane can be seen (fig. 11D). Outer cells of CLA25F embryos presented abundant microvilli and the perivitelline space (PVs) presented less cell debris than CF group (fig. 10A, 10B, 10C, 11A, 11B). Cytoplasmic light vesicles were also present but they seemed less abundant in CLA25F than in CF embryos (fig. 11), in contrast with larger and more abundant dense vesicles in CLA25F embryos (fig. 11C, 11B). Lipid droplets appear to fuse with dense vesicles (fig. 10C, 10D). Large cytosolic areas devoid of organelles were seen in CF (fig. 11D).

Table 10: Post-warming survival (3 sessions) of *in vitro* produced sheep embryo cultured with 25 μ M of *trans*-10 *cis*-12 linoleic acid isomer (CLA25 group) or without (control group) and vitrified by open pulled straws (OPS).

treatment	n	post warming integrity (%)	post warming expansion (%)
CONTROL	15	66.7 \pm 10.0 ^a	80.0 \pm 11.3
25CLA	15	93.3 \pm 10.0 ^b	73.3 \pm 11.3

Data (least square means \pm standard error) within the same columns with different superscripts letters are statistically different ($p \leq 0.05$).

Cryopreserved embryos, CLA25V, classified as grade 1 prior to thawing (fig. 12) presented well preserved cells with defined nucleus and one or more distinct nucleolus. Microvilli are observed in outer cells and abundant well preserved mitochondria, with transverse cristae, are seen in outer and inner cells. Cisternae of endoplasmic reticulum, associated or not to mitochondria or nuclear membrane, were also seen as well as large cytosolic areas devoid of organelles (fig. 12). When compared with grade 1 CV embryos (fig. 13), CLA25V presented better preserved cell structures. In fact, at ultrastructural level, despite some organelles are well preserved (fig. 13A) severe signs of cell lysis are seen in CV embryos. These degenerative signs included disrupted membranes and presence of cell debris (fig 13C). Degenerated cells with loss of organelle structure can also be observed in CF embryos (fig. 13D). Light and dense vesicles can be seen in both groups of vitrified embryos despite large dense vesicles appeared larger and more abundant in CLA25V embryos.

5.3.4 Discussion

This study is the first to report an improved morphological and ultrastructural quality of sheep embryos following *in vitro* culture in 25 μ M dose of *trans*-10, *cis*-12 octadecadienoic acid (CLA) supplemented media. This higher quality was associated to an enhanced embryo production rate and resistance to cryopreservation. In fact the improvement in embryo quality, evaluated morphologically and by post warming

integrity, was confirmed by the ultrastructural evaluation showing a superior preservation of cell membranes and organelles and a minor cellular damage in vitrified/warmed CLA supplemented embryos compared to control (CV group).

As referred, sheep IVP embryos are difficult to cryopreserve and the lower resistance to freezing/thawing has been associated to a more severe cellular damage than the observed in *in vivo* derived embryos (Dattena *et al.*, 2004; Dalcin *et al.*, 2013). These different changes can be attributed to both processes of embryo IVP and cryopreservation. Several methods have been tried to enhance IVP embryos cryoresistance either using different cryopreservation techniques or changing the composition of media and of the IVP embryo itself during *in vitro* production (Seidel Jr, 2006; Pereira and Marques, 2008; Arav, 2014). Indeed the higher susceptibility to cryopreservation has been related to IVP embryo composition, especially the higher lipid content, which can be associated to an increased lipid phase transition and related to ultrastructural changes during cryopreservation (Thompson *et al.*, 1995; Arav *et al.*, 2000; Pereira *et al.*, 2007; Dalcin *et al.*, 2013). Thus, attempts to improve cryosurvival results by the reduction of lipid content and/or composition of IVP embryos prior to cryopreservation have been investigated. Chemical delipidation was applied in embryos of other species with promising results (CLA, Pereira *et al.*, 2007 and 2008, Batista *et al.*, 2014; forskolin, Men *et al.*, 2006; phenazine ethosulfate, Barceló-Fimbres and Seidel Jr, 2007). Herein CLA supplementation improved both sheep IVP embryo production rates and cryosurvival. However, these results were obtained with a 25 μ M dose as our previous data (Romão *et al.*, 2014) showed a harmful action of 100 μ M CLA, the dose applied successfully in bovine embryo production and cryopreservation (Pereira *et al.*, 2007, 2008; Batista *et al.*, 2014). This discrepancy, however, is consistent with other studies reporting varying results of CLA accordingly to the culture system or the CLA isomer used (Gomez *et al.*, 2013; Stinshoff *et al.*, 2014). Data herein presented reveal that sheep embryo culture media supplementation with 25 μ M CLA clearly improved cleavage and D6/7 embryo rates, which were superior compared to control ($p=0.04$ and $p=0.009$ respectively, table 8), as well as blastocyst quality ($p\leq 0.03$, table 9). Despite morphological classification by stereoscopic microscopy is essential for evaluation of embryo quality it remains as one of the most subjective steps of the embryo production process (Aguilar *et al.*, 2002). Ultrastructural evaluation of sheep embryos has been performed in previous studies (Ferrer *et al.*, 1995; Bettencourt *et al.*, 2009; Bettencourt *et al.*, 2014) allowing an accurate characterization of cell structure of the produced embryos. Moreover Bettencourt *et al.* (2009) identified a higher level of ultrastructural cell damage

after cryopreservation using different techniques, associated with different fertility rates after embryo transfer to recipient ewes.

In this study the use of CLA in the culture media improved nuclear and cytoplasmic membrane preservation in fresh embryos presenting also abundant mature mitochondria and microvilli and less cell debris in the perivitelline space compared to CF. These could be related to the effect of CLA during embryo development. Hence the *trans*-10 *cis*-12 CLA isomer is a biologically active compound capable of interfering in different cellular mechanisms and specifically in lipid metabolism reducing the fatty acids up-take, their synthesis and also increasing lipolysis probably through the activation of the peroxisome proliferator-activated receptors (PPARs), cytosolic perilipins and/or key enzymes of lipid metabolism (Pariza *et al.*, 2001, Prates *et al.*, 2014). The exact mechanism through which this isomer influences embryo lipid metabolism is not fully understood. Stinshoff *et al.* (2014) referred that CLA influences bovine embryonic lipid metabolism although their results were not very expressive. Later, Batista *et al.* (2014) reported a reduced abundance of mRNA for the 1-acylglycerol-3-phosphate O-acyltransferase-encoding gene, which is involved in triglycerides synthesis, and the consequent reduction in embryo neutral lipid content in CLA treated embryos. Accordingly, cattle embryos cultured in CLA supplemented media had smaller lipid droplets and fat embryo indexes (Pereira *et al.*, 2007, 2008). It is known that poor quality embryos are characterized by a large number of lipid droplets and a dark appearance as well as immature mitochondria and less well-developed junctional complexes between cells and apical microvilli on the blastomeres (Abe *et al.*, 2002). In addition a higher degree of cell damage with complete cell lysis, disruption of cytoplasmic and nuclear membranes is frequently allied to severe mitochondrial damage in these poor quality embryos either fresh or cryopreserved (Ferrer *et al.* 1995, Cocero *et al.*, 2002). The reduced number of total as well as of mature mitochondria has been associated to the inefficient metabolism of lipids observed in IVP embryos impairing embryo quality (Crosier *et al.*, 2000; Abe *et al.*, 2004). Thus CLA beneficial effect on IVP sheep embryo development and quality reported in the present study may be related to a more efficient lipid metabolism resulting in less lipid droplets accumulation and healthier mitochondria and consequently an enhanced cryosurvival.

Interestingly, besides presenting a better cell structures preservation either in fresh or after thawing, CLA25 embryos had also larger and more abundant dense vesicles. Light and dense vesicles were previously described in IVP and *in vivo* derived embryos (Ferrer *et al.*, 1995; Cocero *et al.*, 2002; Bettencourt *et al.*, 2009; Bettencourt *et al.*, 2014). Although their function is not completely understood, it was proposed that they could be originated from small cisternae of Golgi and endoplasmic reticulum behaving as active

autophagic organelles and representing a maturation process from compact morulae to blastocyst, probably playing a role in the formation of blastocoelic cavity (Bettencourt *et al.*, 2014). In this study it was observed that these dense vesicles were seen to fuse and incorporate lipid compounds (figure 1), being larger and more abundant in embryos produced in culture media containing 25 μ M CLA. Therefore we can hypothesize that these dense vesicles may contribute to the chemical delipidation effectuated by this isomer in IVP embryos. Further studies should be performed to clarify the physiological mechanisms of action of CLA during early embryo development.

In conclusion, the addition of CLA to serum-containing media improved sheep embryo production rates, morphological and ultrastructural quality enhancing cryopreservation survival.

5.3.5 References

- Abe H, Hoshi H 2003. Evaluation of bovine embryos produced in high performance serum-free media. *J Reprod Dev* 49(3), 193-202.
- Abe H, Matsuzaki S, Hoshi H 2002. Ultrastructural differences in bovine morulae classified as high and low qualities by morphological evaluation. *Theriogenology* 57, 1273-1283.
- Abe H, Shiku H, Aoyagi S, Hoshi H 2004. *In vitro* culture and evaluation of embryos for production of high quality bovine embryos. *J. Mamm. Ova Res.* 21, 22-30.
- Aguilar MM, Galina CS, Merchant H, Montiel F, Canseco R, Marquez YC 2002. Comparison of stereoscopy, light microscopy and ultrastructural methods for evaluation of bovine embryos. *Reprod Dom Anim* 37, 341–346.
- Arav A 2014. Cryopreservation of oocytes and embryos. *Theriogenology* 81(1), 96-102.
- Arav A, Pearl M, Zeron Y 2000. Does membrane lipid profile explain chilling sensitivity and membrane lipid phase transition of spermatozoa and oocytes? *Cryo Lett.* 21, 179–186.
- Barceló-Fimbres M, Seidel Jr GE 2007. Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during *in vitro* culture of bovine embryos on embryonic development after cryopreservation. *Mol Reprod Dev* 74, 1395-1405.
- Batista RITP, Raposo NRB, Campos-Junior PHA, Pereira MM, Camargo LSA, Carvalho BC, Gama MAS, Viana JHM 2014. *Trans*-10, *cis*-12 conjugated linoleic acid reduces neutral lipid content and may affect cryotolerance of *in vitro*-produced crossbred bovine embryos. *Journal of Animal Science and Biotechnology*, 5 (33), 1-8.
- Bettencourt EMV, Bettencourt CMV, Chagas e Silva JN, Ferreira P, Oliveira E, Romão R, Rocha A, Sousa M 2014. Ultrastructural characterization of *in vivo*-produced ovine morulae and blastocysts. *Zygote* 30, 1-11.
- Bettencourt EMV, Bettencourt CM, Chagas e Silva JN, Ferreira P, de Matos CP, Oliveira E, Romão RJ, Rocha A, Sousa M 2009. Ultrastructural characterization of fresh and cryopreserved *in vivo* produced ovine embryos. *Theriogenology* 71(6), 947-58.
- Cocero MJ, Moreno Díaz de la Espina S, Aguilar B 2002. Ultrastructural characteristics of fresh and frozen-thawed ovine embryos using two cryoprotectants. *Biology of Reproduction* 66, 1244-1258.
- Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE 2000. Ultrastructural morphometry of bovine compact morulae produced *in vivo* or *in vitro*. *Biology of Reproduction* 62,1459-1465.
- Dalcin L, Silva RC, Paulini F, Silva BD, Neves JP, Lucci CM 2013. Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos. *Cryobiology* 67,137-45.

- Dattena M, Accardo C, Pilchi S, Isachenko V, Mara L, Chessa B, Cappai P 2004. Comparison of different vitrification protocols on viability after transfer of ovine blastocysts *in vitro* produced and *in vivo* derived. *Theriogenology* 62, 481-493.
- Fair T, Lonergan P, Dinnyes A, Cottell D, Hyttel P, Ward FA, Boland MP 2001. Ultrastructure of bovine blastocysts following cryopreservation: effect of method of embryo production on blastocyst quality. *Mol Reprod Dev* 58,186–195.
- Ferrer F, Garcia C, Villar J, Arias M 1995. Ultrastructural study of the early development of the sheep embryo. *Anat Histol Embryol* 24(3),191-196.
- Gómez NA, López AP, Ortiz LF, Ruiz ZT, Olivera M, Tarazona A 2013. Effect of conjugated linoleic acid on sex ratio and quality of *in vitro* produced bovine embryos. *Arch Med Vet* 45, 17-24.
- Hasler JF, Henderson WB, Hurtgen PJ, Zin ZK, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes, JE, Trimmer SA. 1995. Production freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* 42, 141–152.
- Men H, Agca Y, Riley L, Critser JK 2006. Improved survival of vitrified porcine embryos after partial delipitation through chemically stimulated lipolysis and inhibition of apoptosis. *Theriogenology* 66, 2008-2016.
- Pariza MW, Park Y, Cook ME 2001. The biologically active isomers of conjugated linoleic acid. *Prog Lipid Res* 40, 283-298.
- Pereira RM, Marques CC 2008. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank* 9, 267–277.
- Pereira RM, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Chagas e Silva J, Silva Pereira M, Marques CC 2007. Cryo-survival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t,12c CLA). *Animal Reproduction Science* 98, 293-301.
- Pereira RM, Carvalhais I, Pimenta J, Baptista MC, Vasques MI, Horta AEM, Santos IC, Marques MR, Reis A, Silva Pereira M, Marques CC 2008. Biopsied and vitrified bovine embryos viability is improved by *trans*10, *cis*12 conjugated linoleic acid supplementation during *in vitro* embryo culture. *Animal Reproduction Science* 106, 322-332.
- Pollard JW, Leibo SP 1993. Comparative cryobiology of *in vitro* and *in vivo* derived bovine embryos. *Theriogenology* 39, 287.
- Prates EG, Alves S, Marques CC, Baptista MC, Horta AEM, Bessa RJB, Pereira RM. 2013b. Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators *trans*-10, *cis*-12 conjugated linoleic acid (t10,c12 CLA) and forskolin. *In Vitro Cellular & Developmental Biology – Animal* 49, 335-345.
- Prates EG, Marques CC, Baptista MC, Vasques MI, Carolino N, Horta AEM, Charneca R, Nunes JT, Pereira RM 2013a. Fat area and lipid droplets morphology of porcine oocytes during *in vitro* maturation with *trans*-10, *cis*-12 conjugated linoleic acid (t10,c12 CLA) and forskolin. *Animal* 7, 602-609.
- Prates EG, Nunes JT, Pereira RMLN. 2014. A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production. *Mediators of Inflammation*, volume 2014, Article ID 692067, 11 pages. <http://dx.doi.org/10.1155/2014/692067>
- Romão R, Marques CC, Baptista MC, Vasques MI, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Plancha C, Rodrigues P, Pereira RM 2013. Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen. *Small Ruminant Research* 110, 36-41.
- Romão R, Marques, CC, Baptista MC, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Pereira RM 2014. Cryopreservation of *in vitro* produced sheep embryos: effect of different protocols of lipid reduction. (submitted paper).
- SAS Institute. SAS® 9.1.2 for Microsoft Windows. SAS International, Heidelberg, Germany. 2004.
- Seidel Jr GE 2006. Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology* 65: 228-235.

PAPERS SUBMITTED TO PUBLICATION

- Stringfellow DA, Seidel SM, editors 1998. Manual of the International Embryo Transfer Society Savoy II, USA.
- Stinshoff H, Wilkening S, Hanstedt A, Bollwein H, Wrenzycki C 2014. Dimethylsulfoxide and conjugated linoleic acids affect bovine embryo development *in vitro*. *Reprod Fertil Dev* 26(4), 502-10.
- Thibier M, Guérin B 2000. Embryo transfer in small ruminants: the method of choice for health control in germplasm exchanges. *Livest. Prod. Sci.* 62, 253–270.
- Thompson JG 2000. *In vitro* culture and embryo metabolism of cattle and sheep embryos—a decade of achievement. *Anim Reprod Sci* 30, 273–280.
- Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR 1995. Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos. *Biol Reprod.* 53(6),1385-91.
- Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol. Reprod. Dev.* 51, 53–58.

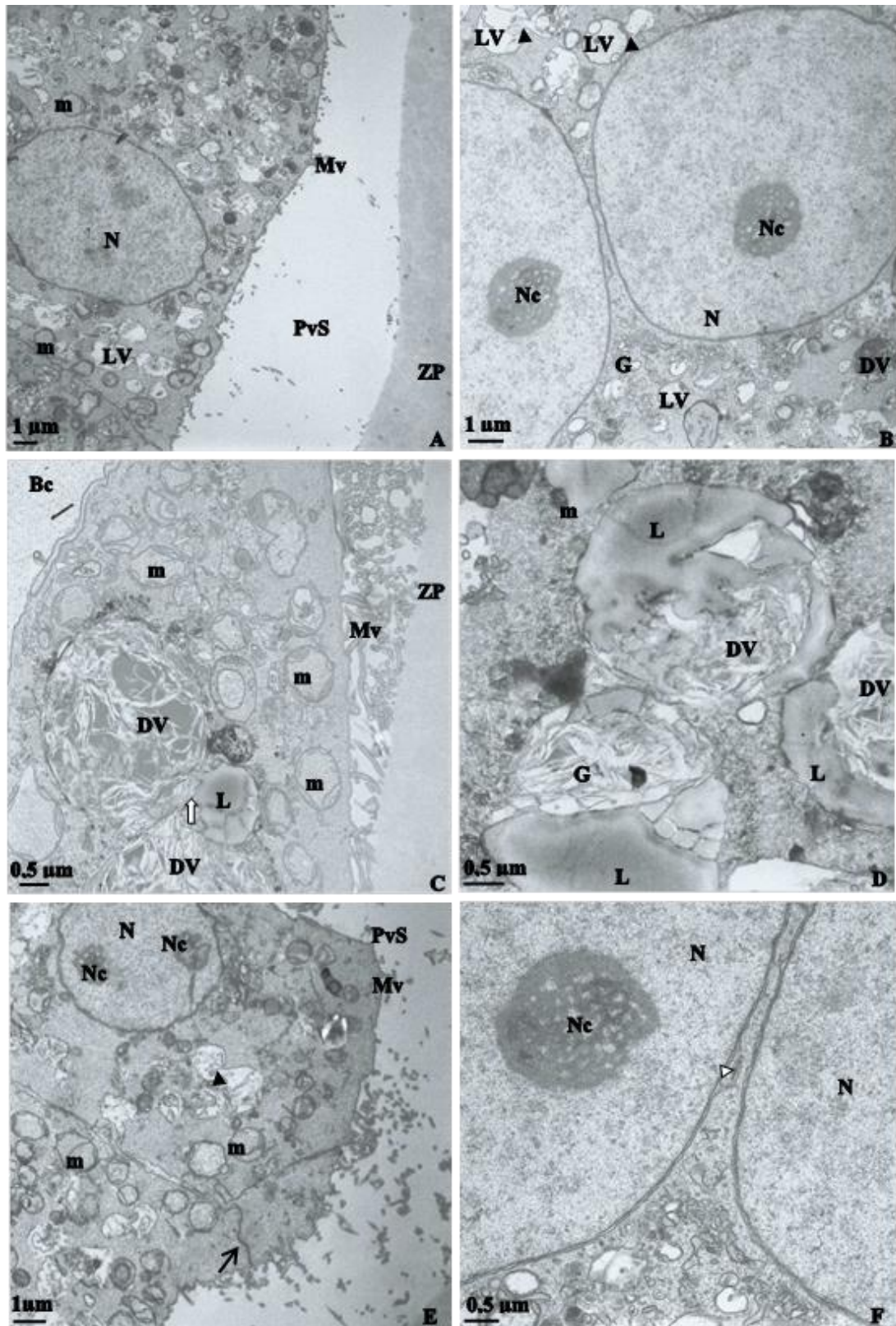


Figure 10: Ultrastructure of fresh sheep blastocysts produced in culture media supplemented with 25 μM of *trans*-10 *cis*-12 linoleic acid isomer (CLA25F). Outer (A,C,E) and inner cells (B,D,F), cells. ZP pellucida (ZP), microvilli (Mv), perivitelline space (PvS), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light (LV), dense vesicles (DV), lipid droplets (L), fusion of lipid droplets with dense vesicles (white open arrow), fusion of light vesicles (black arrow head), Golgi complexes (G), nucleus (N), nucleolus (Nc), blastocoel cavity (Bc) .

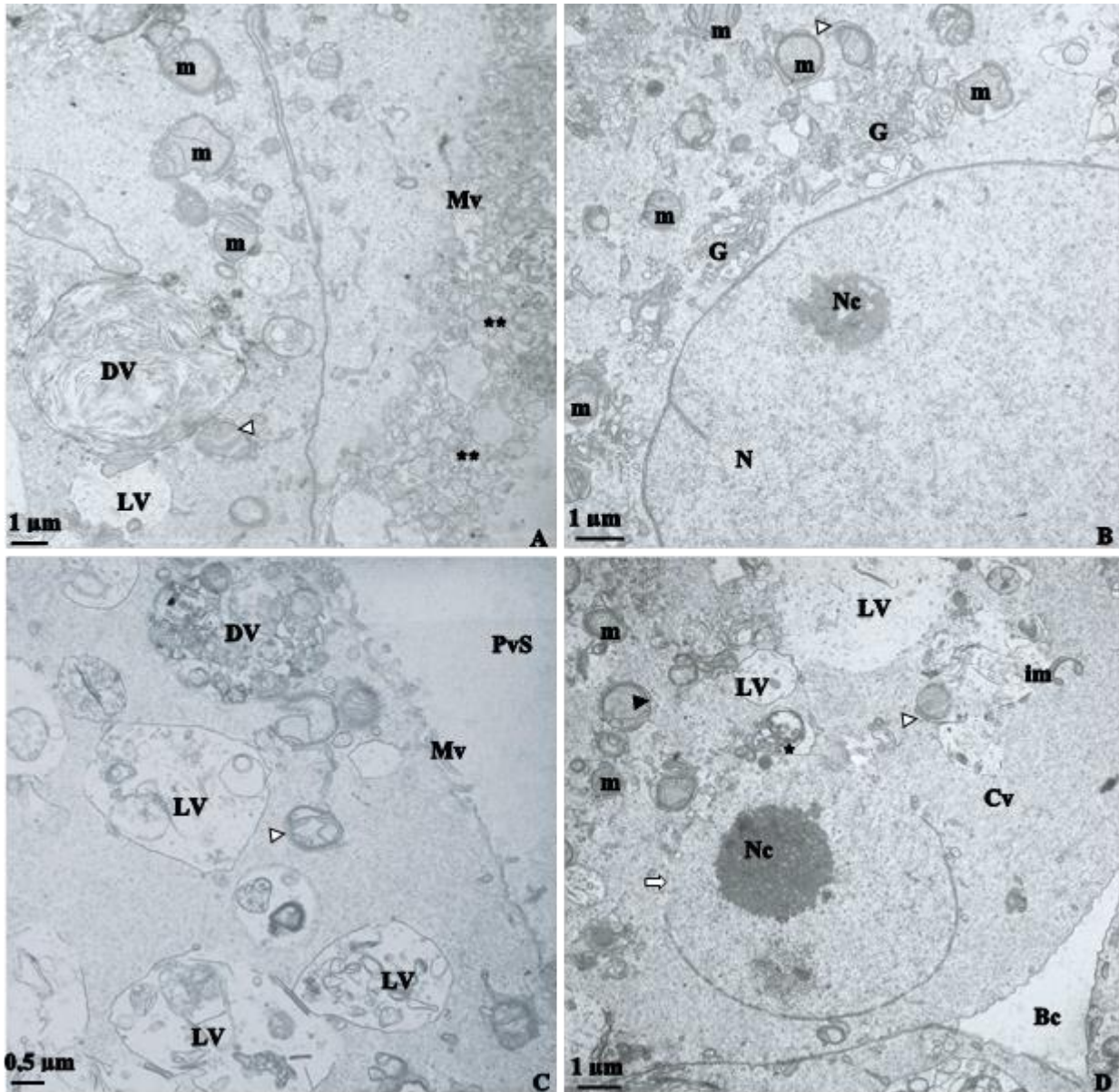


Figure 11: Ultrastructure of fresh *in vitro* produced sheep blastocysts (control fresh, CF). Outer (A,C) and inner cells (B,D), cells. Microvilli (Mv),perivitelline space (PvS); mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), disrupted nuclear membrane (white open arrow), ** cell debris cytosolic areas devoid of organelles (Cy) blastocoel cavity (Bc).

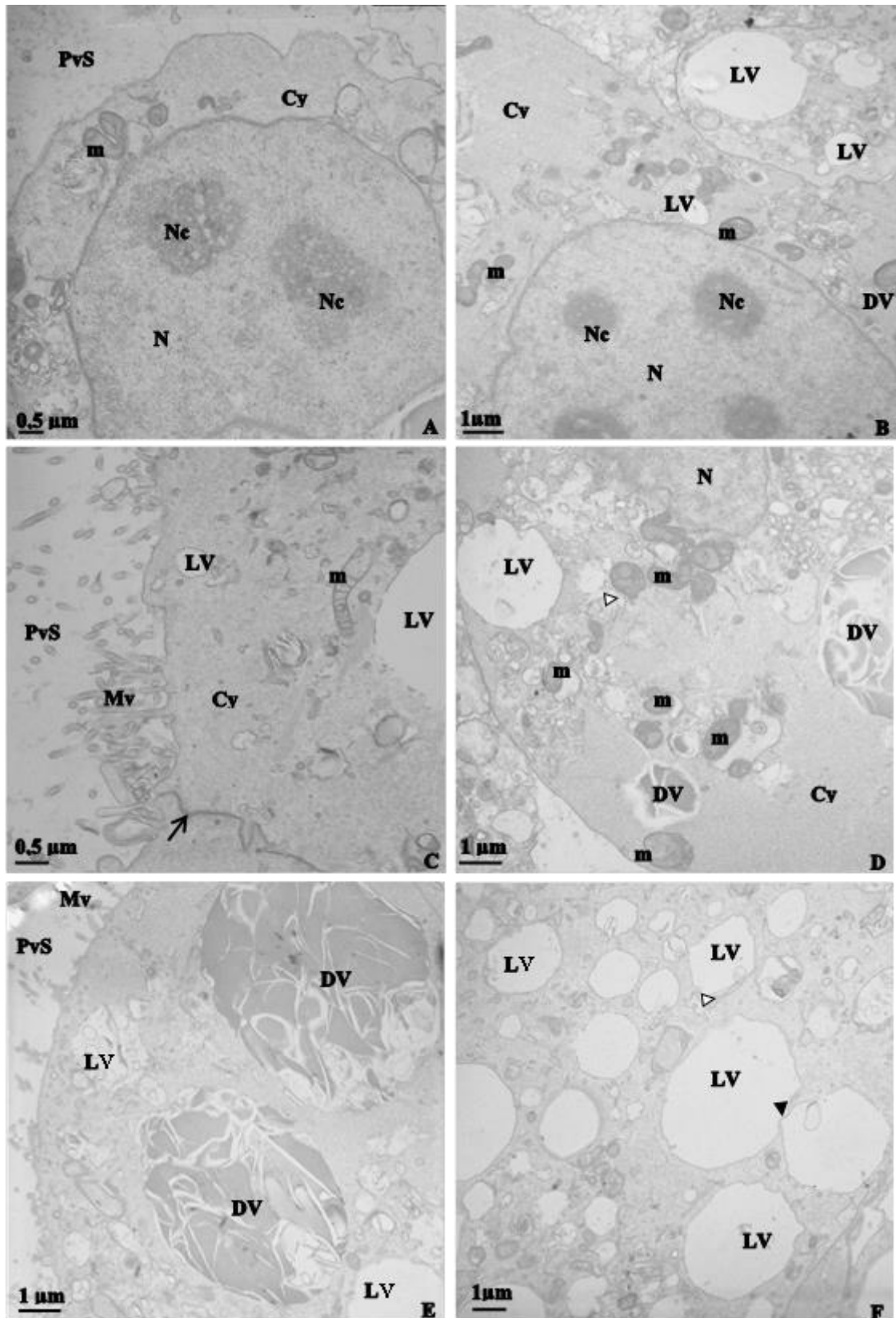


Figure 12: Ultrastructure of OPS vitrified sheep blastocysts produced in culture media supplemented with 25 μM of *trans*-10 *cis*-12 linoleic acid isomer (CLA25V) and fixed 1h after warming. Perivitelline space (PvS), microvilli (Mv), blastocoel cavity (b), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), fusion of light vesicles (black arrow head), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), cytosolic areas devoid of organelles (Cy).

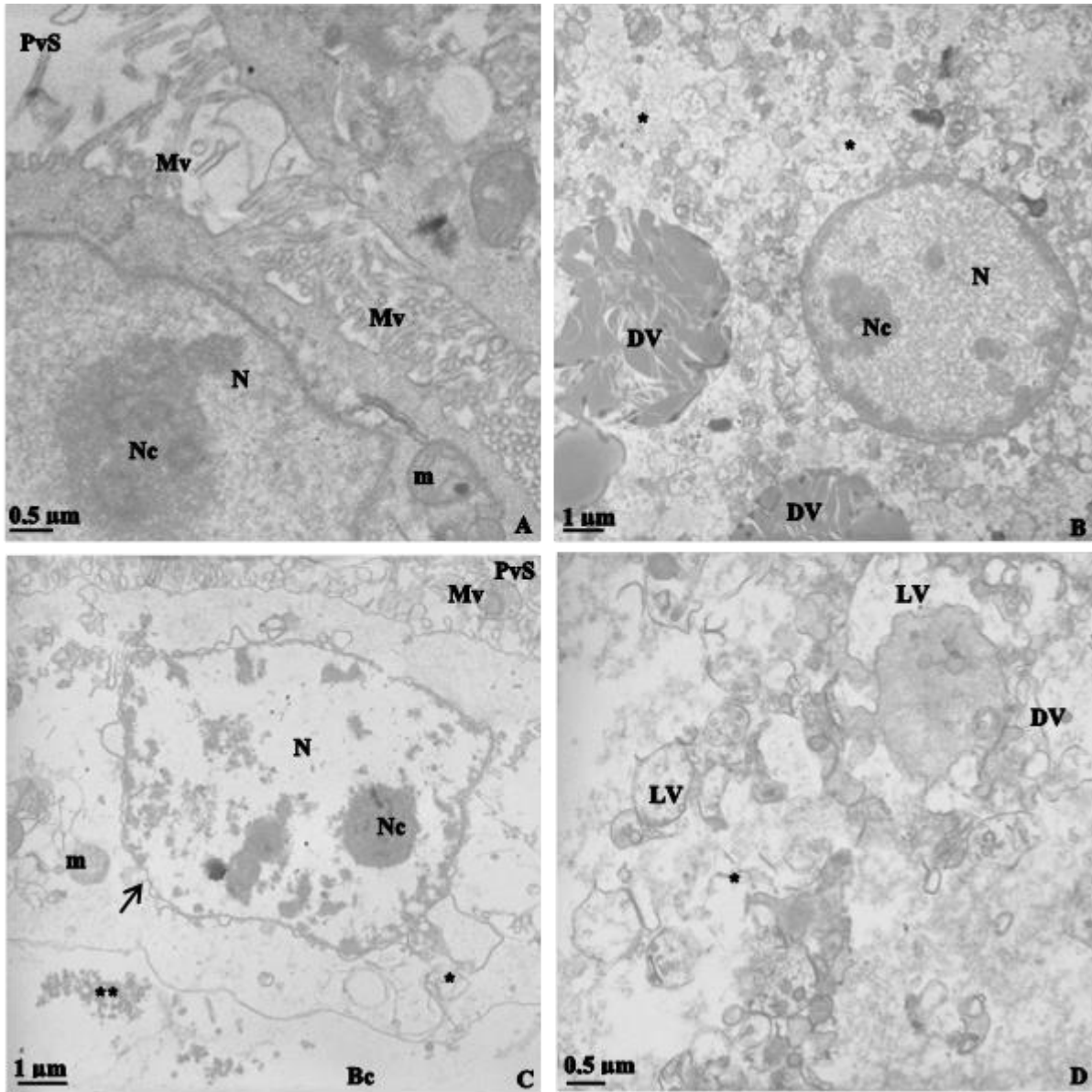


Figure 13: Ultrastructure of OPS vitrified *in vitro* produced sheep blastocysts (control vitrified, CV), fixed 1h after warming. Perivitelline space (PvS), microvilli (Mv), blastocoel cavity (b), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (LV), dense vesicles (DV), Golgi complexes (G), nucleus (N), nucleolus (Nc), *, cell lysis, ** cell debris, disrupted nuclear membrane (black arrow).

6 Discussion and conclusions

After completing this thesis, summarized in this humble book, it is important to look back trying to understand the contribution of our efforts to upgrade our skills, as this is the goal of a PhD, and also to assure if we were able to afford some positive outputs in our demanding society. In the range of our professional activity in Animal Production and in Research and Education, attention should be paid first of all to the profitable development of our world and population. Livestock production in the 21st century is somewhat the eye of the hurricane, trying to attend many issues from the economic and technological productivity scope, to environmental, societal or ethical concerns. The demanding of the world growing population is now asking for “sustainable intensification” (Wathes *et al.*, 2013), what means producing more and more but ensuring the protection of natural resources and animal welfare guarantee, beyond human ethical considerations, in which food supply is included.

It is impossible to predict the following steps in animal production worldwide but it is manifest that nowadays we are dealing with the nutritional needs of a huge growing population, mainly in emerging economies, along with the disruptions felt in some critical areas of the planet as are climate changes, pollution, emergence of new diseases or irreparable loss of biodiversity (Conraths *et al.*, 2011). It is stated that to overcome the huge future challenges in Animal Production it is important that researchers and technicians work closely with farmers so that existing and newly knowledge – associated with better education and greater social and economic equality – could be managed to reach the needs and expectations of Humankind (Galli and Lazzari, 2008; Godfray *et al.*, 2010).

In vitro embryo production and cryopreservation are challenging techniques that have been growing worldwide in all species. The general goal is to improve results allowing its future use as an essential tool in livestock reproductive medicine. Likewise in the Human species where results must guarantee an application to enable an expansion of the clinical services, needed to solve the emerged infertility problems (Konc *et al.*, 2005), in animal reproduction the main target should be that cryo-technologies achievements allow approaching the viability of fresh embryos.

Sheep embryo technology is less applied than in other animal species (e.g. bovine) consequently to economical and scientific limitations. It is very clear that, in the next few years, these techniques will have a central role in small ruminant production answering to genetic and reproductive needs. Economic justification for sheep biotechnologies is based on the relation cost/benefit. These techniques should be used mainly for genetic improvement, embryo production and cryopreservation. However as in other species, developmental

DISCUSSION AND CONCLUSIONS

engineering techniques including bisection, sexing, nuclear transplantation, intracytoplasmic injection, and delipation may be also used to improve the productivity and availability of embryos (Ushijima, 2005).

In the present work we focused in the cryopreservation of sheep IVP embryos trying to find new perspectives for improving the feasible results that limits its application. In a first approach we demonstrated that supplementing the maturation medium of oocytes with FSH, EGF and pyruvate allied to the use of fresh ram semen for IVF could improve the quality of produced embryos. The role of these compounds had been previously demonstrated (Paria and Dey, 1990; Wang *et al.*, 2009). Moreover a synergy between EGF and FSH during oocyte maturation was already identified in pig (Uhm *et al.*, 2010). Regarding ram semen, although there was not an increase in production rates, in accordance with other authors (Pugh *et al.*, 1991; Lehloenya *et al.*, 2010), it was found that the use of fresh semen in IVF can improve sheep embryo developmental competence by enhancing morphological blastocyst quality (Romão *et al.*, 2013). A synergic effect with the previously referred supplementation of the maturation medium in sheep embryo quality seems to occur. This may be the result of the production of hyaluronic acid (HA), which is promoted by FSH during maturation contributing to the process of spermatozoa capacitation and acrosome reaction during IVF and subsequent developmental competence (Gutnisky *et al.*, 2007).

As previously indicated cryopreservation of IVP sheep embryos reduces its survival rates. In order to understand these detrimental effects, we compared for the first time the ultrastructural changes occurring in these IVP embryos and their *in vivo* derived counterparts. Differences in embryo ultrastructure in fresh and after vitrification/warming by OPS were assessed. The results showed that fresh IVP sheep embryos had comparable ultrastructure to their *in vivo* counterparts, although these *in vivo* embryos had more microvilli, more visible smooth endoplasmic reticulum and Golgi complexes and increased number of mature mitochondria displaying peripheral and transverse cristae. IVP embryos appear to have many immature mitochondria, which has been associated to an inefficient metabolism of lipids previously observed in this source of embryos (Dorland *et al.*, 1994; Crosier *et al.*, 2000). These differences were also described in cattle (Crosier *et al.*, 2000; Rizos *et al.*, 2002a). In both groups of fresh embryos (*in vivo* and IVP) it was evident the presence of large light vesicles, as well as medium and large dense vesicles, according to what was previously described in sheep (Ferrer *et al.*, 1995; Bettencourt *et al.*, 2009, 2014). After cryopreservation higher damage in cell structures were observed in IVP embryos. These alterations vary from small changes in grade 2 blastocysts (BL) at semithin score to large alterations in grade 3 BL. The main alterations associated to cryopreservation included disruption of cellular membranes and poor intracellular preservation.

These changes have been already advocated as responsible for the reduced cryotolerance of IVP embryos (Crosier *et al.*, 2000; Rizos *et al.*, 2002b). Only *in vivo* derived vitrified embryos scored as grade 1 had well preserved cytoplasm, intercellular junctions and a well-defined nucleus and nucleolus, presenting the same organelles described for fresh embryos.

Signs of cell degeneration, with disruption of nuclear membrane and poor intracellular preservation as well as loss of microvilli, non-defined cellular limits, poor cellular preservation and presence of cellular debris were observed in OPS vitrified IVP embryos. The described changes can be associated with impaired cell constitution that negatively influences metabolism, being responsible for the reduced viability of these embryos. Although not specifically quantified in our study, it appeared that IVP embryos had a higher amount of lipid droplets. This fact can be related to the malfunction of mitochondria and lipid dynamics with sequential problems in the energy demands of cells that are essential for embryo development (Dunning *et al.*, 2010; Leese, 2012). Moreover, the high lipid content in embryos of different species has been appointed as one of the major causes of the reduced cryotolerance of embryos, further exacerbated in IVP embryos (Thompson *et al.*, 1995; Dattena *et al.*, 2000; Abe *et al.*, 2004; Pereira *et al.*, 2007, Pereira and Marques, 2008). For this reason attempts have been made to promote delipidation, either in embryos or in oocytes (Nagashima *et al.*, 1994; Ushijima *et al.*, 1999; Diez *et al.*, 2001; Pereira *et al.*, 2007; Prates *et al.*, 2013). Following this idea we planned several experiments in order to promote embryo delipidation, being applied in sheep for the first time. Physical methods namely centrifugation, in the presence or absence of a cytoskeleton stabilizer – cytochalasin D – were used. These methods had been previously tried in other species (Dobrinsky *et al.*, 1997; Tominaga *et al.*, 2000; Franco and Hansen, 2006; Li *et al.*, 2009). Also for a chemical approach CLA was supplemented to embryo culture media. This strategy had been also successfully applied in bovine (Pereira *et al.*, 2007; 2008; Accorsi, 2008, Batista *et al.*, 2014). Our results demonstrated that different protocols of lipid reduction improved the cryotolerance of IVP sheep embryos. In fact, the use of cytochalasin D associated with centrifugation previously to vitrification in OPS enhanced the blastocyst expansion rates after vitrification/warming, mainly due to an enhanced number of expanded embryos with an excellent blastocoele. Moreover the supplementation of culture medium with 25 and 50 μM CLA enabled higher IVP sheep embryo production rates. Previously, it was demonstrated that CLA reduces fatty acids uptake, synthesis and deposition in IVP embryos (Pereira *et al.*, 2007, 2008, Batista *et al.*, 2014). Although the action of CLA in embryos has not been clearly elucidated, it seems that this isomer can interfere with lipid metabolism, decreasing fatty acid synthesis, up-take and deposition and increasing

DISCUSSION AND CONCLUSIONS

lipolysis through the regulation of adipogenic genes, the activation of the peroxisome proliferator-activated receptors (PPARs), cytosolic perilipins and/or key enzymes of lipid metabolism (Pariza *et al.*, 2001; Harvey *et al.*, 2002; Prates *et al.*, 2014; Stinshoff *et al.*, 2014). Additionally, once inside the embryo, CLA may follow the mitochondrial β -oxidation to produce energy or to synthesize fatty acids to be used during development (Harvey *et al.*, 2002; Reis *et al.*, 2003; Prates *et al.*, 2014). The central role of mitochondria in lipid metabolism and the formation and the necessary neutralization of ROS are essential for embryo developmental competence (Harvey *et al.*, 2002; Barceló-Fimbres and Seidel Jr, 2007). As previously highlighted, major changes were observed in sheep embryo ultrastructure namely in the amount and quality of mitochondria.

The observed ultrastructural differences in IVP embryos and especially in IVP sheep embryos cryopreserved can justify the lower viability of these embryos after cryopreservation. Moreover even fresh IVP embryos showed a decrease of total mitochondria and an increase in the amount of immature ones, when compared to *in vivo* derived fresh embryos. As demonstrated by Crosier *et al.* (2000) immature mitochondria have a reduced ability to metabolize lipids and produce ATP impairing embryo developmental competence. This problem seems to be overcome by CLA supplementation during embryo development.

To achieve a deeper insight into the beneficial effects of CLA on preventing cellular cryoinjuries, a delineation was designed to study the ultrastructural changes observed either in fresh or in vitrified sheep IVP embryos cultured with or without CLA supplementation. As in the preceding study, 25 μ M CLA supplementation improved cleavage and also D 6/7 embryo rates. The latter dose is lower than the dose successfully applied in bovine embryo production and cryopreservation (Pereira *et al.*, 2007, 2008; Batista *et al.*, 2014), but our previous results had demonstrated the adverse effect of 100 μ M CLA concentration in ovine species. In addition, CLA presence also improved embryo quality, mainly with an increase of grade 1 embryos production. Again, CLA supplementation showed some beneficial effects on post warming embryo characteristics. These embryos presented a higher rate of post warming integrity, although no differences in post warming expansion rate were observed.

Ultrastructural features of cryopreserved IVP sheep embryos cultured in the presence of CLA were described for the first time in the present study. Besides the above mentioned higher embryo production rates and resistance to cryopreservation, vitrified/warmed embryos cultured with CLA showed also an improved morphological quality revealing superior preservation of cell membranes and organelles as well as an inferior cellular damage when compared to control. Previous papers have commented the ultrastructural

alterations that occur in cryopreserved sheep embryos (Cocero *et al.*, 2002; Bettencourt *et al.*, 2009). These changes and also the presence of some structures as light and dense vesicles, previously described (Ferrer *et al.*, 1995; Cocero *et al.*, 2002; Bettencourt *et al.*, 2009, 2014), have been pointed to be probably related with lipid metabolism. However their function is not well elucidated. In this experiment dense vesicles seem to fuse and incorporate lipid compounds, being larger and more abundant in embryos produced in culture media containing 25 μ M CLA. It is possible that these vesicles may be involved in chemical delipidation mediated by CLA. Thus the induced reduction in lipid content can improve sheep embryo cryotolerance.

We demonstrated the effect of delipidation, either by physical or chemical methods, in the production rates, morphological quality and ultrastructural characteristics of IVP sheep embryos. We strongly believe that the latter technology can open a new optimistic research window in this species. In the future, manipulation of delipidation can trigger other research teams to study this biotechnology as one of the main steps to get a breakthrough in sheep IVP embryos cryopreservation. Moreover, research in this particular and also in other interconnected subjects, needs to be continued, either to improve or to test new cryopreservation methods, always adapting them to IVP in sheep.

Although we know that much effort is still needed for implementing routine use of IVP embryo cryopreservation in sheep, we believe that our work and discussion had contributed to some scientific knowledge on the subject. This work should be continued in the near future and for that we hope for political, economic and scientific aspiration as well as inspiration.

7 References²

- Abe H, Hoshi H. Evaluation of bovine embryos produced in high performance serum-free media. *Journal of Reproduction and Development* 2003;49(3):193-202.
- Abe H, Matsuzaki S, Hoshi H. Ultrastructural differences in bovine morulae classified as high and low qualities by morphological evaluation. *Theriogenology* 2002; 57:1273-1283.
- Abe H, Shiku H, Aoyagi S, Hoshi H. *In vitro* culture and evaluation of embryos for production of high quality bovine embryos. *J. Mamm. Ova Res.* 2004;21:22-30.
- Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H. Ultrastructure of bovine embryos developed from *in vitro*-matured and – fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. *Molecular Reproduction and Development* 1999;53:325-335.
- Accardo C, Dattena M, Pilichi S, Mara L, Chessa B, Cappai P. Effect of recombinant human FSH and LH on *in vitro* maturation of sheep oocytes; embryo development and viability. *Animal Reproduction Science* 2004;81:77-86.
- Accorsi MF. Assessment of bovine embryos grown *in vitro* in the presence of fatty acids and their survival after cryopreservation. Dissertation – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, Brasil, 2008.
- Alabart JL, Lahoz B, Folch J, Sánchez P, Echegoyen E, Cocero MJ. Effect of a defined or undefined *in vitro* maturation medium on cleavage rate and embryo development in sheep. 26th Annual Meeting AETE, Kuopio, Finland, 2000.
- Amiridis GS, Cseh S. Assisted reproductive technologies in the reproductive management of small ruminants. *Animal Reproduction Science* 2012;130:152-161.
- Amstislavsky SY, Trukshin IS. Cryobanking mammalian embryos: priorities and the optimal choice of reproductive technologies. *Russian Journal of Developmental Biology* 2010;41(1):13-23.
- Arav A, Pearl M, Zeron Y. Does membrane lipid profile explain chilling sensitivity and membrane lipid phase transition of spermatozoa and oocytes? *Cryo Lett.* 2000;21:179–186.
- Arav A, Revel A, Nathan Y, Bor A, Gacitua H, Yavin S, Gavish Z, Uri M, Elami A. Oocyte recovery, embryo development and ovarian function after cryopreservation and transplantation of whole sheep ovary. *Human Reproduction* 2005;20(12):3554-3559.
- Arav A, Zvi R. Do chilling injury and heat stress share the same mechanism of injury in oocytes? *Mol Cell Endocrinol.* 2008; 30;282(1-2):150-2.
- Arav A. Cryopreservation of oocytes and embryos. *Theriogenology* 2014;81(1):96-102.

² These list includes all bibliographic citations included in Preamble, Introduction and Discussion and Conclusion.

- Assumpção MEOA, Milazzotto MP, Simões R, Nicacio AC, Mendes CM, Mello MRB, Visintin JA. *In vitro* survival of *in vitro*-produced bovine embryos cryopreserved by slow freezing, fast freezing and vitrification. *Anim. Reprod.* 2008;5(3/4):116-120.
- Bączkowski T, Kurzawa R, Głąbowski W. Methods of embryo scoring in *in vitro* fertilization. *Reproductive Biology* 2004;4(1):5-22.
- Bâge R. Collection and preservation of female gametes and embryos. *Farm animal reproduction: conserving local genetic resources. Proceedings from a minisymposium at Lithuanian Veterinary Academy, Kaunas, Lithuania*, pp 22, 2003.
- Balasubramanian S, Rho G-J. Effect of chilling on the development of *in vitro* produced bovine embryos at various cleavage stages. *Journal of Assisted Reproduction and Genetics* 2006;23(2):55-61.
- Baldassarre H, Furnus CC, de Matos DG, Pessi H. *In vitro* production of sheep embryos using laparoscopic folliculocentesis: alternative gonadotrophin treatments for stimulation of oocyte donors. *Theriogenology* 1996;45:707-714.
- Baldassarre H, Wang B, Kafidi N, Keefer C, Lazaris A, Karatzas CN. Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and *in vitro* embryo production technologies. *Theriogenology* 2002;57:275-284.
- Baptista MC, Marques CC, Vasques MI, Pereira RM, Barbas JP, Horta AEM. Implementação em Portugal da técnica de fertilização *in vitro* em ovinos. *Resumos do Congresso de Ciências Veterinárias, SPCV, Oeiras, Portugal*: 465-466, 2002.
- Barceló-Fimbres M, Seidel Jr GE. Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during *in vitro* culture of bovine embryos on embryonic development after cryopreservation. *Mol Reprod Dev* 2007;74:1395-1405.
- Bari F, Khalid M, Wolf B, Haresign W, Murray A, Merrell B. The repeatability of superovulatory response and embryo recovery in sheep. *Theriogenology* 2001;56:147-155.
- Baril G, Traldi AL, Cognié Y, Leboeuf B, Beckers JF, Mermillod P. Successful direct transfer of vitrified sheep embryos. *Theriogenology* 2001;56:299-305.
- Batista RITP, Raposo NRB, Campos-Junior PHA, Pereira MM, Camargo LSA, Carvalho BC, Gama MAS, Viana JHM. *Trans*-10, *cis*-12 conjugated linoleic acid reduces neutral lipid content and may affect cryotolerance of *in vitro*-produced crossbred bovine embryos. *Journal of Animal Science and Biotechnology* 2014;5(33):1-8.
- Bernardi ML. Produção *in vitro* de embriões ovinos. *Acta Scientiae Veterinariae* 2005;33(1):1- 16.
- Bettencourt EMV, Bettencourt CM, Silva JNCE, Ferreira P, de Matos CP, Oliveira E, Romão RJ, Rocha A, Sousa M. Ultrastructural characterization of fresh and cryopreserved *in vivo* produced ovine embryos. *Theriogenology* 2009;71(6):947-58.

REFERENCES

- Bettencourt, EM, Bettencourt, CM, Chagas e Silva, J, Ferreira, P, Matos, CP, Romão, RJ, Rocha, A. Fertility rates following the transfer of ovine embryos cryopreserved using three protocols. *Small Ruminant Research* 2009b;82:112-116.
- Bettencourt EMV, Bettencourt CMV, Chagas e Silva JN, Ferreira P, Oliveira E, Romão R, Rocha A, Sousa M. Ultrastructural characterization of *in vivo*-produced ovine morulae and blastocysts. *Zygote*, 2014;30:1-11.
- Betteridge K. Farm animal embryo technologies: achievements and perspectives. *Theriogenology* 2006;65: 905-913.
- Betts DH, King WA. Genetic regulation of embryo death and senescence. *Theriogenology* 2001;55:171-191.
- Betts DH, Madan P. Permanent embryo arrest: molecular and cellular concepts. *Molecular Human Reproduction* 2008;14 (8):445-453.
- Birler S, Pabuccuoglu S, Alkan S, Ak K, Evecen M, Ileri IK. Effects of different maturation and culture media on IVF of sheep oocytes. *Pakistan Vet. J.* 2001;21(2):100-102.
- Blanco MR, Demyda S, Moreno Milán M, Genero E. Developmental competence of *in vivo* and *in vitro* matured oocytes: a review. *Biotechnology and Molecular Biology Review* 2011;6(7):155-165.
- Bogliolo L, Ariu F, Fois S, Rosati I, Zedda MT, Leoni G, Succu S, Pau S, Ledda S. Morphological and biochemical analysis of immature ovine oocytes vitrified with or without cumulus cells. *Theriogenology* 2007;68:1138-1149.
- Cho, S-R, Cho, S-K, Lee, S-L, Lee, H-J, Choe, S-Y, Rho, G-J. Enhanced cryosurvival of bovine blastocysts produced *in vitro* in serum-free medium. *Journal of Assisted Reproduction and Genetics* 2002;19(10):487.
- Cocero MJ, Alabart JL, Hammami S, Martí JI, Lahoz B, Sánchez P, Echegoyen E, Beckers JF, Folch J. The efficiency of *in vitro* ovine embryo production using an undefined or a defined maturation medium is determined by the source of the oocyte. *Reprod Dom Anim* 2011 ;46:463-470.
- Cocero MJ, Lopez Sebastian A, Barragán ML, Picazo A. Differences on post-thawing survival between ovine morulae and blastocysts cryopreserved with ethylene glycol and glycerol. *Criobiology* 1996;33: 502-507.
- Cocero, MJ, Moreno Díaz de la Espina S, Aguilar B. Ultrastructural characteristics of fresh and frozen-thawed ovine embryos using two cryoprotectants. *Biology of Reproduction* 2002;66:1244-1258.
- Cognié Y, Baril G, Poulin N, Mermillod P. Current status of embryo technologies in sheep and goat. *Theriogenology* 2003;59:171-188.
- Cognié Y, Baril G. Le point sur la production et le transfert d'embryons obtenus *in vivo* et *in vitro* chez la brebis et la chèvre. *INRA Prod. Anim.* 2002;15(3):199-207.
- Cognié Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. *Reproduction, Fertility and Development* 2004;16:437-445.
- Conraths FJ, Schwabenbauer K, Vallat B, Meslin FX, Füssel AE, Slingenbergh J, Mettenleiter TC. Animal health in the 21st century-a global challenge. *Prev Vet Med.* 2011;102(2):93-7.
- Çörekçi Ş, Yilmaz A. A comparative study on the growth and survival characteristics of lambs produced by the transfer of *in vitro* produced (IVP) embryos. *Turk J Vet Anim Sci* 2004;28:831-835.

- Cotterill M, Catt SL, Picton HM. Characterisation of the cellular and molecular responses of ovine oocytes and their supporting somatic cells to pre-ovulatory levels of LH and FSH during *in vitro* maturation. *Reproduction* 2012;144:195–207.
- Cox JF, Alfaro V. *In vitro* fertilization and development of OPU derived goat and sheep oocytes. *Reproduction in Domestic Animals* 2007;42:83-87.
- Crocomo LF, Marques Filho WC, Landim Alvarenga FC, Bicudo SD. Peculiaridades da coleta de oócitos para produção *in vitro* de embriões ovinos. *Rev. Bras. Reprod. Anim.*, Belo Horizonte, 2012;36(1):25-31.
- Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine compact morulae produced *in vivo* or *in vitro*. *Biology of Reproduction* 2000;62:1459-1465.
- Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine blastocysts produced *in vivo* or *in vitro*. *Biology of Reproduction* 2001;64:1375-1385.
- Cuello C, Berthelot F, Delaleu B, Venturi E, Pastor LM, Vazquez JM, Roca J, Martinat-Botte F, Martinez EA. The effectiveness of the stereomicroscopic evaluation of embryo quality in vitrified–warmed porcine blastocysts: an ultrastructural and cell death study. *Theriogenology* 2007; 67:970–982.
- Czlonkowska M, Eysymont U, Guskiewicz A, Kossakowski M, Dziak J. Birth of lamb after *in vitro* maturation, fertilisation, and co-culture with oviductal cells. *Mol Rep Dev* 1991;30:34-38.[abstract]
- Dalcin L, Lucci CM. Criopreservação de embriões de animais de produção: princípios criobiológicos e estado atual. *Rev. Bras. Reprod. Anim.*, Belo Horizonte 2010;34(3):149-159.
- Dalcin L, Silva RC, Paulini F, Silva BD, Neves JP, Lucci CM. Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos. *Cryobiology* 2013;67(2):137-45.
- Dattena M, Accardo C, Pilchi S, Isachenko V, Mara L, Chessa B, Cappai P. Comparison of different vitrification protocols on viability after transfer of ovine blastocysts *in vitro* produced and *in vivo* derived. *Theriogenology* 2004;62:481-493.
- Dattena M, Ptak G, Loi P, Cappai P. Survival and viability of vitrified *in vitro* and *in vivo* produced ovine blastocysts. *Theriogenology* 2000;53:1511-1519.
- Dattena, M, Isachenko, V, Alabart, JL, Folch, J, Accardo, C, Cappai, P. Comparison between two embryo transfer methods of vitrified sheep blastocysts. *Proceedings of the 17th scientific meeting AETE, Lyon, pp 113, 2001.*
- De AK, Malakar D, Akshey YS, Jena MK, Garg S, Dutta R, Sahu S. *In vitro* development of goat (*Capra hircus*) embryos following cysteamine supplementation of the *in vitro* maturation and *in vitro* culture media. *Small Ruminant Research* 2011;96:185-190.
- Deleuze S, Goudet G. Cysteamine supplementation of *in vitro* maturation media: a review. *Reprod Dom Anim* 2010;45:476-482.

REFERENCES

- Dieleman SJ, Hendriksen PJM, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TAM, Niemann H, Gadella BM, Bevers MM, Vos PLAM. Effect of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology* 2002;57:5–20.
- Diez C, Heyman Y, Le Bourhis D, Guyader-Joly C, Degrouard J, Renard JP. Delipidating *in vitro*-produced bovine zygotes: effect on further development and consequences for freezability. *Theriogenology* 2001;55:923-936.
- Dinnyes A, Meng Q, Polgar Z, Boonkuso D, Somfai T. Cryopreservation of mammalian embryos. *Acta Sci Vet* 2006;34(supl.1):171-190.
- Dobrinsky JR, Long CR Johnson LA. Stability of microfilaments during swine embryo cryopreservation. *Theriogenology* 1997;47(1):343[abstract].
- Dobrinsky JR, Nagashima H, Purse VG, Schreier LL, Johnson LA. Cryopreservation of morula and early blastocyst stage swine embryos: birth of litters after embryo transfers. *Theriogenology* 2001;55:303 [abstract].
- Dobrinsky JR, Pursel VG, Long CR, Johnson LA. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. *Biol Reprod* 2000;62:564-570.
- Dobrinsky JR. Advancements in cryopreservation of domestic animal embryos. *Theriogenology* 2002;57:285-302.
- Dobrinsky JR. Cellular approach to cryopreservation of embryos. *Theriogenology* 1996;45:17-26.
- Dorland M, Gardner DK, Trounson AO. Serum in SOF causes mitochondrial degeneration in ovine embryos. *J Reprod Fertil* 1994;13:70.
- Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol Reprod* 2010;83(6):909-18.
- Fabian D, Gjørret JO, Berthelot F, Martinat-Botte F, Maddox-Hyttel P. Ultrastructure and cell death of *in vivo* derived and vitrified porcine blastocysts. *Molecular Reproduction and Development* 2005;70:155-165.
- FAO. Secondary guidelines for development of national farm animal genetic resources management plans. Management of small populations at risk. FAO, Rome, Italy, 1998.
- <http://www.fao.org/ag/againfo/programmes/pt/lead/toolbox/Indust/sml-popn.pdf> (acceded on 12.05.2014)
- Farin PW, Crosier AE, Farin CE. Influence of *in vitro* systems on embryo survival and fetal development in cattle. *Theriogenology* 2001;55:151-170.
- Faustino LR, Santos RR, Silva CMG, Pinto LC, Celestino JJH, Campello CC, Figueiredo JR, Rodrigues APR. Goat and sheep ovarian tissue cryopreservation: Effects on the morphology and development of primordial follicles and density of stromal cell. *Animal Reproduction Science* 2010;122:90-97.
- Ferrer F, Garcia C, Villar J, Arias M. Ultrastructural study of the early development of the sheep embryo. *Anat Histol Embryol* 1995;24(3):191-6.
- Fogarty NM, Maxwell WM, Eppleston J, Evans G. The viability of transferred sheep embryos after long-term cryopreservation. *Reprod Fertil Dev.* 2000;12(1-2):31-7.

- Folch, J, Olivera, J, Aguilar, B, Alabart, JL, Sanchez, P, Echegoyen, E, Cocero, MJ. Resultados obtenidos em la transferència de embriones dentro del programa genético de la UPRA carnes Oviaragon. Resúmenes de las XXV Jornadas SEOC: 559-561, 2000.
- Franco M, Hansen PJ. Effects of hyaluronic acid in culture and cytochalazin B treatment before freezing on survival of cryopreserved bovine embryos produced *in vitro*. *In Vitro Cell. Dev. Biol. – Animal* 2006;42:40-44.
- Freitas VJF, Andrade MLL, Cajazeiras JB, Luz JV. *In vitro* embryo production in small ruminants raised in northeastern Brazil. *Acta Scientiae Veterinariae*. 2007;35(Supl. 3):s781-s786.
- Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard M-A, Schellander K, Tesfaye D. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative *in vivo* and *in vitro* culture conditions. *Biology of Reproduction* 2012;87(4),100:1–13.
- Gajda B, Smorąg V. Oocyte and embryo cryopreservation – state of art and recent developments in domestic animals. *Journal of Animal and Feed Sciences* 2009;18:371–387.
- Galli C, Lazzari G. The manipulation of gametes and embryos in farm animals. *Reprod Dom Anim* 2008;43(suppl. 2):1–7.
- Gama LT. Animal genetic resources and sustainable development in the Mediterranean region. In: Ramalho Ribeiro JM, Horta AEM, Mosconi C, Rosati A (eds) *Animal products from the Mediterranean area*. EAAP pub.119. Wageningen Academic Publishers:127-135, 2006.
- Gao, D, Critser, JK. Mechanisms of cryoinjury in living cells. *ILAR J*. 2000;41(4):187-96.
- Garcia-Garcia RM, Gonzalez-Bulnes A, Domínguez V, Veiga-Lopez A, Cocero MJ. Culture of early stage ovine embryos to blastocyst enhances survival rate after cyopreservation. *Theriogenology* 2005;63:2233-2242.
- Garcia-Garcia RM, Gonzalez-Bulnes A, Domínguez V, Veiga-Lopez A, Cocero MJ. Survival of frozen-thawed sheep embryos cryopreserved at cleavage stages. *Cryobiology* 2006;52:108-113.
- Gibbons A, Bonnet FP, Cueto MI, Catala M, Salamone DF, Gonzalez-Bulnes A. Procedure for maximizing oocyte harvest for *in vitro* embryo production in small ruminants. *Reprod Dom Anim* 2007;42:423–426.
- Gibbons A, Bonnet FP, Cueto MI, Salamone D, Catala M. Colheita de oócitos guiada por laparoscopia em caprinos e ovinos. *Acta Sci Vet* 2008;36(supl. 2):223-230.
- Gibbons A, Cueto M. Reproductive biotechnologies for genetic improvement in sheep. *Rev. Bras. Reprod. Anim., Belo Horizonte* 2011;35(2):180-185.
- Gibbons, A, Cueto, MI, Pereyra Bonnet, F (2011). A simple vitrification technique for sheep and goat embryo cryopreservation. *Small Ruminant Research* 2011;95:61–64.
- Godfray HCJ, Crute IR, Haddad L, Lawrence D, Muir JF, Nisbett N, Pretty J, Robinson S, Toulmin C, Whiteley R. The future of the global food system. *Phil. Trans. R. Soc. B* 2010;365:2769–2777.
- Green, RE, Santos, BFS, Sicherle, CC, Landim-Alvarenga, FC, Bicudo, SD. Viability of OPS vitrified sheep embryos after direct transfer. *Reprod Dom Anim* 2009;44:406-410.

REFERENCES

- Guérin B, Le Guennea B, Thibier M. A secure health status associated with the production and trade of *in vitro* derived cattle embryos. *Livestock Production Science* 2000;62:271–285.
- Guler A, Poulin N, Mermillod P, Terqui M, Cognié Y. Effect of growth factors, EGF and IGF-1, and estradiol on *in vitro* maturation of sheep oocytes. *Theriogenology* 2000;54:209-218.
- Gupta MK, Lee H-T. Cryopreservation of oocytes and embryos by vitrification. *Korean. J. Reprod. Med.* 2010;37(4):267-291.
- Gusmão AL. State-of-the-art in the transcervical embryo collection in goats and sheep. *Acta Scientiae Veterinariae* 2011;39(Suppl 1):37- 42.
- Gutnisky C, Dalvit GC, Pintos LN, Thompson JG, Beconi MT, Cetica PD. Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte *in vitro* maturation, fertilisation and embryo development. *Reprod. Fertil. Dev.* 2007;19:488-497.
- Harvey AJ, Kind KL, Thompson JG. REDOX regulation of early embryo development. *Reproduction* 2002;123:479-486.
- Harvey AJ. The role of oxygen in ruminant preimplantation embryo development and metabolism. *Animal Reproduction Science* 2007;98:113–128.
- Hasler J. Bovine embryo transfer: are efficiencies improving? *Proceedings, Applied Reproductive Strategies in Beef Cattle*. Sioux Falls, USA: 319-338, 2012.
- Henson EL. *In situ* conservation of livestock and poultry. *FAO Animal Production health paper*, 1992. <http://www.fao.org/docrep/004/T0559E/T0559E00.htm#TOC> [accessed 20.11.2013]
- Hernandez-Ledezma JJ, Mathialagan N, Villanueva C, Sikes JD, Roberts RM. Expression of bovine trophoblast interferons by *in vitro*-derived blastocysts is correlated with their morphological quality and stage of development. *Mol Reprod Dev.* 1993;36(1):1-6.
- Hochi S, Semple E, Leibo SP. Effect of cooling and warming rates during cryopreservation on survival of *in vitro*-produced bovine embryos. *Theriogenology* 1996;46:837-847.
- Hollinshead FK, Evans G, Evans KM, Catt SL, Maxwell WMC, O'Brien JK. Birth of lambs of a pre-determined sex after *in vitro* production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa. *Reproduction* 2004;127:557-568.
- Holm P, Walker SK, Seamark RF. Embryo viability, duration of gestation and birth weight in sheep after transfer of *in vitro* matured and *in vitro* fertilized zygotes cultured *in vitro* or *in vivo*. *J Reprod Fertil* 1996;107:175-181.
- Hosseini SM, Hajian M, Asgari V, Foroanfar M, Abedi P, Nasr-Esfahani MH. Novel approach of differential staining to detect necrotic cells in preimplantation embryos. *Iranian Journal of Fertility and Sterility*, 2007;1(3):103-106.
- Ikeda S, Imai H, Yamada M. Apoptosis in cumulus cells during *in vitro* maturation of bovine cumulus-enclosed oocytes. *Reproduction* 2003;125:369–376.

- Isachenko V, Alabart JL, Dattena A, Nawroth F, Cappai, P, Isachenko E, Cocero, MJ, Olivera, J, Roche A, Accardo C, Kriovokharchneko A, Folch J. New technology for vitrification and field (microscope-free) warming and transfer of small ruminant embryos *Theriogenology* 2003;59(5-6):1209-1218.
- Kartberg A-J; Hambiliki F; Arvidsson T; Stavreus-Evers A; Svalander P. Vitrification with DMSO protects embryo membrane integrity better than solutions without DMSO. *Reproductive BioMedicine Online* 2008;17(3):378-384.
- Kasai, M, Mukaida, T. Cryopreservation of animal and human embryos by vitrification. *Reproductive BioMedicine Online* 2004;9(2):164-170.
- Kelly J, Kleemann D, Kuwayama M, Walker S. Pregnancy rates for *in vitro* and *in vivo* produced ovine embryos vitrified using the minimum volume cooling cryotop method. *Reproduction Fertility and Development* 2005;17(2):194-194.
- Khalifa T, Lymberopoulos, A. Changeability of sperm chromatin structure during liquid storage of ovine semen in milk-egg yolk and soybean lecithin based extenders and their relationships to field-fertility. *Cell Tissue Bank* 2013;14:687-698.
- Khurana NK, Niemann H. Effects of cryopreservation on glucose metabolism and survival of bovine morulae and blastocyst derived *in vitro* or *in vivo*. *Theriogenology* 2000;54:313-326.
- Kim YM, Uhm SJ, Gupta MK, Yang JS, Lim JG, Das ZC, Heo YT, Chung H-J, Kong I-K, Kim N-H, Lee T, Ko DH. Successful vitrification of bovine blastocysts on paper container, *Theriogenology* 2012;78:1085–1093.
- Knijn HM, Gjørret JO, Vos PLAM, Hendriksen PJM, van der Weijden BC, Maddox-Hyttel P, Dieleman SJ. Consequences of *in vivo* development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos. *Biology of Reproduction* 2003;69:1371-1378.
- Konc J, Cseh S, Varga E, Kriston R, Kanyó K. Cryopreservation of oocytes and embryos in Human Assisted Reproduction. *J. Reproduktionsmed. Endokrinol* 2005;2(4):251-258.
- Kong IK, Lee SI, Cho SG. Comparison of open pulled straw (OPS) vs glass micropipette (GMP) vitrification in mouse blastocysts. *Theriogenology* 2000;53:1817-1826.
- Kuwayama M. Evidence-based embryo cryopreservation. *J. Mamm. Ova Res.* 2005;22:28-32.
- Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology* 2007;67:73-80.
- Lapa M, Marques CC, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Pereira RM. Fatty acid profile of oocytes and blastocysts during *in vitro* bovine embryo production. *Reprod Dom Anim* 2005;40(4):365.
- Lazzari G, Colleoni S, Lagutina I, Crotti G, Turini P, Tessaro I, Brunetti D, Duchi R, Galli C. Short-term and long-term effects of embryo culture in the surrogate sheep oviduct versus *in vitro* culture for different domestic species. *Theriogenology* 2010;73:748-757.
- Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruij T, Niemann H, Galli C. Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biology of Reproduction* 2002;67:767-775.

REFERENCES

- Leese HL. Metabolism of the preimplantation embryo: 40 years on. *Reproduction* 2012;143:417-427.
- Lehloenya KC, Mahoete N, Greyling JPC, Nedambale TL. Effect of breed and frozen–thawed ram semen on *in vitro* fertilization and ovine embryonic development. *Reprod. Fertil. Dev.* 2010;23:170.
- Leibo SP, Martino A, Kobayashi S, Pollard JW. Stage-dependent sensitivity of oocytes and embryos to low temperatures. *Animal Reproduction Science* 1996;42:45-53.
- Leibo SP, Pollard JW, Martino A. Chilling and freezing sensitivity of “reassembled” *in vitro*-derived bovine embryos. *Theriogenology* 1995;43(1):265[abstract].
- Leoni GG, Berlinguer F, Succu S, Bebbere D, Mossa F, Madeddu M, Ledda S, Bogliolo L, Naitana S. A new selection criterion to assess good quality ovine blastocysts after vitrification and to predict their transfer into recipients. *Molecular Reproduction and Development* 2008;75:373-382.
- Li R, Murphy CN, Spate L, Wax D, Isom C, Rieke A, Walters EM, Samuel M, Prather RS. Production of piglets after cryopreservation of embryos using a centrifugation-based method for delipitation without micromanipulation. *Biology of Reproduction* 2009;80:563-571.
- Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. Potential importance of vitrification in reproductive medicine. *Biology of Reproduction* 2002;67:1671-1680.
- Lin TA, Chen CH, Sung LY, Carter MG, Chen YE, Du F, Ju JC, Xu J. Open-pulled straw vitrification differentiates cryotolerance of *in vitro* cultured rabbit embryos at the eight-cell stage. *Theriogenology* 2011;75:760-768.
- Liu J, Westhusin M, Long C, Johnson G, Burghardt R, Kraemer D. Embryo production and possible species preservation by nuclear transfer of somatic cells isolated from bovine semen. *Theriogenology* 2010;74(9):1629-35.
- Loneragan P, Fair T. The ART of studying early embryo development: Progress and challenges in ruminant embryo culture. *Theriogenology* 2014;81:49-55.
- Machado S, Marques CC, Baptista MC, Horta AEM, Pereira RM. Ovarian tissue cryopreservation for preservation of Portuguese animal genetic resources. *Proc. VIII Congresso Ibérico sobre os Recursos Genéticos Animais, SPREGA/SERGA, Évora:27, 2012.*
- Mara L, Casub S, Cartac A, Dattena M. Cryobanking of farm animal gametes and embryos as a means of conserving livestock genetics. *Animal Reproduction Science* 2013;138:25-38.
- Martínez AG, Matkovic M. Cryopreservation of ovine embryos: slow freezing and vitrification. *Theriogenology* 1998;49:1039-1049.
- Martínez AG, Valcácel A, Furnus CC, de Matos DG, Iorio G, de las Heras MA. Cryopreservation of *in vitro*-produced ovine embryos. *Small Rum Res* 2006;63(3):288-296.
- Massip A, Mermillod P, Dinnyes A. Morphology and biochemistry of *in vitro* produced bovine embryos: implications for their cryopreservation. *Hum. Reprod.* 1995;10:3004-3011.
- Massip A. Cryopreservation of embryos of farm animals. *Reprod. Dom. Anim.* 2001;36:49-55.

- McCreath KJ, Howcroft J, Campbell KHS, Colman A, Schnieke AE, Kind AJ. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* 2000;405:1066-1069.
- McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JS, Speake BK. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *J Reprod Fertil.* 2000;118(1):163-70.
- Merdassi G, Mazoyer C, Guerin JF, Saad A, Salle B, Lornage J. Examination of viability and quality of ovarian tissue after cryopreservation using simple laboratory methods in ewe. *Reproductive Biology and Endocrinology* 2011;9:1-7.
- Mermillod P, Locatelli Y, Dalbiès-Tran R, Uzbekova S, Baril G, Guignot F, Perreau C, Poulin N, Touzé JL, Pannetier S, Schmaltz B, Cognié Y. *In vitro* production of ruminant embryos: results, limits and perspectives. Symposium COA/INRA Scientific Cooperation in Agriculture, Tainan, Taiwan, ROC, 2006.
- Mezzalira A, Vieira AD, Barbieri DP, Machado MF, Thaler Neto A, Bernardi ML, Silva AM, Rubin MIB. Vitricificação de oócitos e embriões bovinos produzidos *in vitro* e expostos à citocalasina B. *Braz. J. Vet. Res Anim. Sci.* 2002;39(5):260-265.
- Mochida K, Hasegawa A, Li M-W, Fray MD, Kito S, Vallelunga JM, Lloyd KCK, Yoshik Ai, Obata Y, Ogura A. High osmolality vitrification: a new method for the simple and temperature-permissive cryopreservation of mouse embryos. *PLoS ONE* 2013;8(1):e49316:1-8.
- Morató R, Mogas T. New device for the vitrification and in-straw warming of *in vitro* produced bovine embryos. *Cryobiology* 2014;68:288-293.
- Mullen SF, Fahy GM. A chronologic review of mature oocyte vitrification research in cattle, pigs, and sheep. *Theriogenology* 2012;78:1709-1719.
- Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG, Seamark RF, Nottle MB. Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. *Biol Reprod* 1994;51:618-622.
- Nedambale TL, Dinnyés A, Yang X, Tian C. Bovine blastocyst development *in vitro*: timing, sex, and viability following vitrification. *Biology of Reproduction* 2004;71:1671-1676.
- O'Brien JK, Catt SL, Ireland KA, Maxwell WMC, Evans G. *In vitro* and *in vivo* developmental capacity of oocytes from prepubertal and adult sheep. *Theriogenology* 1997 47:1433-1443.
- O'Brien JK, Dwarthe D, Ryan JP, Maxwell WM, Evans G. Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. *Reprod. Fertil. Dev.* 1996;8:1029-1037.
- Ohboshi S, Fujihara N, Yoshida T, Tomagane H. Ultrastructure of bovine *in vitro*-produced blastocysts cryopreserved by vitrification. *Zygote* 1998;6:17-26.
- Papadopoulos S, Rizos D, Duffy P, Wade M, Quinn K, Boland MP, Lonergan P. Embryo survival and recipient pregnancy rates after transfer of fresh or vitrified, *in vivo* or *in vitro* produced ovine embryos. *Animal Reproduction Science* 2002;74:35-44.

REFERENCES

- Paria BC, Dey SK. Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors. *Proc. Natl. Acad. Sci. USA* 1990;80:4756-4760.
- Pariza MW, Park Y, Cook ME. The biologically active isomers of conjugated linoleic acid. *Prog Lipid Res* 2001;40:283-298.
- Pereira RM, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Chagas e Silva J, Silva Pereira M, Marques CC. Cryo-survival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t, 12c CLA). *Anim. Reprod. Sci.* 2007;98:293-301.
- Pereira RM, Marques CC. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank*, 2008;9:267-277.
- Pereira RM, Mesquita P, Batista M, Baptista MC, Barbas JP, Pimenta J, Santos IC, Marques MR, Vasques MI, Silva Pereira M, Santos Silva F, Oliveira Sousa MC, Fontes CM, Horta AEM, Prates JA, Marques CC. Doppel gene polymorphisms in Portuguese sheep breeds: insights on ram fertility. *Animal Reproduction Science* 2009;114:157-166.
- Peris SI, Morrier A, Dufour M, Bailey JL. Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. *J. Androl.* 2004;25:224-233.
- Perry G. Statistics of embryo collection and transfer in domestic farm animals. 22nd annual report of Data Retrieval Committee. IETS, 2013.
- Prates EG, Alves S, Marques CC, Baptista MC, Horta AEM, Bessa RJB, Pereira RM. Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators *trans*-10, *cis*-12 conjugated linoleic acid (t10,c12 CLA) and forskolin. *In Vitro Cellular & Developmental Biology – Animal* 2013;49:335-345.
- Prates EG, Nunes JT, Pereira RMLN. A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production. *Mediators of Inflammation* 2014, Art. 692067:1-11; <http://dx.doi.org/10.1155/2014/692067>
- Ptak G, Dattena M, Loi P, Tischner M, Cappai P. Ovum pick-up in sheep: efficiency of *in vitro* embryo production, vitrification and birth of offspring. *Theriogenology* 1999;52:1105-1114.
- Pugh PA, Fukui Y, Tervit HR, Thompson JG. Developmental ability of *in vitro* matured sheep oocytes collected during the nonbreeding season and fertilized *in vitro* with frozen ram semen. *Theriogenology* 1991;36:771-778.[abstract]
- Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* 1985;313:573-575.[abstract]
- Rall WF, Meyer TK. Zona fracture damage and its avoidance during the cryopreservation of mammalian embryos. *Theriogenology* 1989;31:683-692.
- Reis A, Rooke JA, McCallum GJ, Staines ME, Ewen M, Lomax MA, McEvoy, TG. Consequences of exposure to serum, with or without vitamin E supplementation, in terms of the fatty acid content and viability of bovine blastocysts produced *in vitro*. *Reprod Fertil Dev* 2003;15:275-284.

- Ricci G, Perticarari S, Boscolo R, Montico M, Guaschino S, Presani G. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. *Fertil Steril*. 2009;91(2):632-638.
- Rizos D, Fair T, Papadopoulos S, Boland M, Lonergan P. Developmental, qualitative and ultrastructural differences between ovine and bovine embryos produced *in vivo* and *in vitro*. *Mol Reprod Dev* 2002a;62:320-327.
- Rizos D, Gutierrez-Adan A, Moreira P, O'Meara C, Fair T, Evans ACO, Boland MP, Lonergan P. Species-related differences in blastocyst quality are associated with differences in relative mRNA transcription. *Molecular Reproduction and Development* 2004;6:381-386.
- Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* 2002b;61:234-248.
- Rodríguez C, Anel L, Alvarez M, Anel E, Boixo JC, Chamorro CA, de Paz P. Ovum pick-up in sheep: a comparison between different aspiration devices for optimal oocyte retrieval. *Reprod Dom Anim* 2006;41:106-113.
- Romão R, Marques CC, Baptista MC, Vasques MI, Barbas JP, Horta AEM, Carolino N, Bettencourt E, Plancha C, Rodrigues P, Pereira RM. Evaluation of two methods of *in vitro* production of ovine embryos using fresh or cryopreserved semen. *Small Ruminant Research* 2013;110:36-41.
- Rooke JA, McEvoy TG, Ashworth CJ, Robinson JJ, Wilmut I, Young LE, Sinclair KD. Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology* 2007;67:639-647.
- Sanches BV, Marinho LSR, Filho BDO, Pontes JHF, Basso AC, Meirinhos MLG, Silva-Santos KC, Ferreira CR, Seneda MM. Cryosurvival and pregnancy rates after exposure of IVF-derived *Bos indicus* embryos to forskolin before vitrification. *Theriogenology* 2013;80:372-377.
- Saragusty J, Arav A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction* 2011;141:1-19.
- Seider Jr, GE. Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology* 2006;65:228-235.
- Shabankareh HK, Sarsaifi K, Mehrannia T. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. *J Assist Reprod Genet* 2011;28:531-537.
- Shamsuddin M, Rodriguez-Martinez H. Fine structure of bovine blastocysts developed either in serum-free medium or in conventional coculture with oviduct epithelial cells. *J Vet Med A* 1994;41:307-316.
- Shirazi A, Ahmadi E, Jadidi M, Shams-Esfandabadi N, Heidari B. Acephalous lamb from an *in vitro*-produced sheep embryo. *The Canadian Veterinary Journal* 2009;50(5):501-505.
- Shirazi A, Shams-Esfandabadi N, Hosseini SM, Karimi I. The presence of cumulus cells on nuclear maturation of sheep oocytes during *in vitro* maturation. *Small Rumin Res* 2007;68:291-295.

REFERENCES

- Shirazi A, Soleimani M, Karimi M, Nazari H, Ahmadi E, Heidari B. Vitrification of *in vitro* produced ovine embryos at various developmental stages using two methods. *Cryobiology* 2010;60(2):204-10.
- Smith GD Silva CASE. Developmental consequences of cryopreservation of mammalian oocytes and embryos. *Reprod. BioMed. Online* 2004;9:171–178.
- Stangl M, Kühholzer B, Besenfelder U, Brem G. Repeated endoscopic ovum pick-up in sheep. *Theriogenology* 1999;52:709-716.
- Steel R, Hasler JF. Pregnancy rates from transfer of fresh and frozen Holstein and Jersey embryos. *Reprod Fertil Dev* 2004;16:182–3. [abstract]
- Stinshoff H, Wilkening S, Hanstedt A, Bollwein H, Wrenzycki C. Dimethylsulfoxide and conjugated linoleic acids affect bovine embryo development *in vitro*. *Reprod Fertil Dev* 2014;26(4):502-10.
- Stringfellow DA, Givens MD. Epidemiologic concerns relative to *in vivo* and *in vitro* production of livestock embryos. *Animal Reproduction Science* 2000;60-61:629–642.
- Stringfellow DA, Seidel Jr. SM, editors. *Manual of the International Embryo Transfer Society Savoy II, USA, 1998.*
- Suttiyotin P, Thwaites CJ. Evaluation of ram semen motility by a swim-up technique. *Journal of Reproduction and Fertility* 1993;97:339-345.
- Thibier M, Guérin B. Embryo transfer in small ruminants: the method of choice for health control in germplasm exchanges. *Livestock Production Science* 2000;62:253-270.
- Thibier M. Embryo transfer: a comparative biosecurity advantage in international movements of germplasm. *Rev. Sci. Tech. Off. Int. Epiz.* 2011;30(1):177-188.
- Thibier M. Stabilization of numbers of *in vivo* collected embryos in cattle but significant increases of *in vitro* bovine produced embryos in some parts of the world. *Data Retrieval Committee Annual Report. IETS Newslett* 2004;22(4):12–9.
- Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos. *Biology of Reproduction* 1995;53:1385-1391.
- Thompson JG. *In vitro* culture and embryo metabolism of cattle and sheep embryos—a decade of achievement. *Anim. Reprod. Sci.* 2000;30:273-280.
- Tominaga K, Shimizu M, Ooyama S, Isaïke Y. Effect of lipid polarization by centrifugation at different developmental stages on post-thaw survival of bovine *in vitro* produced 16-cell embryos. *Theriogenology* 2000 53:1669-1680.
- Tominaga K. Cryopreservation and sexing of *in vivo*- and *in vitro*-produced bovine embryos for their practical use. *J Reprod Dev.* 2004;50:29-38.
- Tsujii H, Khandoker Y, Hamano K. Lipid in mammalian embryo development. *J. Mamm. Ova Res.*, 2001;18:73-80.
- Tveden-Nyborg P, Peura TT, Hartwich KM, Walker SK, Maddox-Hyttel P. Morphological characterization of pre- and peri-implantation *in vitro* cultured, somatic cell nuclear transfer and *in vivo* derived ovine embryos. *Reproduction* 2005;130:681-694.

- Uhm SJ, Gupta MK, Yang JH, Chung H-J, Min TS, Lee HT. Epidermal growth factor can be used in lieu of follicle-stimulating hormone for nuclear maturation of porcine oocytes *in vitro*. *Theriogenology* 2010;73:1024-1036.
- Ushijima H, Akiyama K, Tajima T. Classification of morphological changes based on the number of cleavage divisions in bovine embryos. *Journal of Reproduction and Development*, 2009;55(1):83-87.
- Ushijima H, Yamakawa H, Nagashima H. Cryopreservation of bovine pre-morula-stage *in vitro* matured/*in vitro* fertilized embryos after delipidation and before use in nucleus transfer. *Biology of Reproduction* 1999;60:534-539.
- Ushijima H. Application study of developmental engineering for livestock production. *J. Reprod. Dev.* 2005;51:15–22.
- Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol. Reprod. Dev.* 1998;51:53-58.
- Vajta G, Hyttel P, Callesen H. Morphological changes of *in-vitro*-produced bovine blastocysts after vitrification, in-straw direct rehydration, and culture. *Molecular Reproduction and Development* 1997;48:9-17.
- Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reproductive BioMedicine Online* 2006;12(6):779-796.
- Van Soom A, Tanghe S, De Pauw I, Maes D, de Kruif A. Function of the cumulus oophorus before and during mammalian fertilization. *Reprod Dom Anim* 2002;37:144-151
- Van Soom A, Vanroose G; de Kruif A. Blastocyst evaluation by means of differential staining: a practical approach. *Reprod. Dom. Anim.* 2001;36:29-35.
- Wang LM, Feng HL, Ma YZ, Cang M, Li HJ, Yan Zh, Zhou P, Wen JX, Bou S, Liu DJ. Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. *Anim. Reprod. Sci.* 2009;114:99-108.
- Wani AR, Khan MZ, Sofi KA, Lone FA, Malik AA, Bhat FA. Effect of cysteamine and epidermal growth factor (EGF) supplementation in maturation medium on *in vitro* maturation, fertilization and culturing of embryos in sheep. *Small Ruminant Research* 2012;106:160-164.
- Wani NA, Wani GM, Khan MZ, Salahudin S. Effect of oocyte harvesting techniques on *in vitro* maturation and *in vitro* fertilization in sheep. *Small Ruminant Research* 2000;36:63-67.
- Wani NA. *In vitro* maturation and *in vitro* fertilization of sheep oocytes. *Small Ruminant Research* 2002;44:89-95.
- Wathes CM, Buller H, Maggs H, Campbell ML. Livestock Production in the UK in the 21st Century: a perfect storm averted? *Animals* 2013;3:574-583.
- Willadsen SM, Polge C, Rowson, LEA, Moor, RM. Deep freezing of sheep embryos. *J. Reprod. Fert.* 1976;46:151-154.
- Willadsen SM. Factors affecting the survival of sheep embryos during deep-freezing and thawing. In: *The Freezing of Mammalian Embryos*. Ciba Foundation, Symposium, 52 (New Series). Elsevier Excerpta Medica/North-Holland, Amsterdam: 175-201, 1977. [abstract]
- Wilmot I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997;385:810–813.

REFERENCES

- Woods EJ, Benson JD, Agca Y, Critser JK. Fundamental cryobiology of reproductive cells and tissues. *Cryobiology* 2004;48:146-156.
- Yao Y-C, Qi M-Y, Lu M-H, Wang S-M, Li W, Han H-B. Long-term cryopreservation had no adverse effect on viability of embryos and their offspring in sheep. *Animal Reproduction Science* 2012;136(1):42-46.
- Yu XL, Deng, W, Liu, FJ, Li, YH, Li, XX, Zhang, YL, Zan, LS. Closed pulled straw vitrification of *in vitro*-produced and *in vivo*-produced bovine embryos. *Theriogenology* 2010;73:474-479.
- Zehmer JK, Huang Y, Peng G, Pu J, Anderson RG, Wand Liu P. A role for lipid droplets in inter-membrane lipid traffic. *Proteomics* 2009;9:914–921.
- Zeinoaldini S, Jafari Z, Sarmast F, Torbati E, Davachi ND. Different harvesting techniques used in ovine *in vitro* embryo production. *Scimetr.* 2013;1(1):1-5.
- Zhou P, Liu DJ, Canga M, Maa YZ, Yang DS, Li HJ, Wang LM, Boua S, Feng HL. TGF α and EGFR in ovine preimplantation embryos and effects on development. *Animal Reproduction Science* 2008;104:370-381.



Contactos:

Universidade de Évora
Instituto de Investigação e Formação Avançada - IIFA
Palácio do Vimioso | Largo Marquês de Marialva, Apart. 94
7002-554 Évora | Portugal
Tel: (+351) 266 706 581
Fax: (+351) 266 744 677
email: iifa@uevora.pt