Cryopreservation of semen and embryos of Portuguese sheep breeds

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Ex situ Conservation Group- Webinar on semen cryopreservation in small ruminants

10-11 June 2025, Uppsala, Sweden

REPÚBLICA PORTUGUESA | AGRICULTURA E ALIMENTAÇÃO

iniav National Institute for Agrarian and Veterinary Research

Summary

INIAV Coordinating Researcher, DVM, MSc, PhD, and Aggregation

- Head of the Animal Reproduction area of INIAV's Biotechnology and Genetic Resources Unit
- Head of INIAV's Embryology Laboratory
- Member of the Management Committee of the Portuguese Animal Germplasm Bank
- Member of the Work Group of ex situ Conservation of the European Regional Focal Point for Animal Genetic Resources (ERFP) – FAO
- Lecturer of Theriogenology I and II on Veterinary Medicine course
- **rosa.linoneto@iniav.pt**

PORTUGAL *BIODIVERSITY*

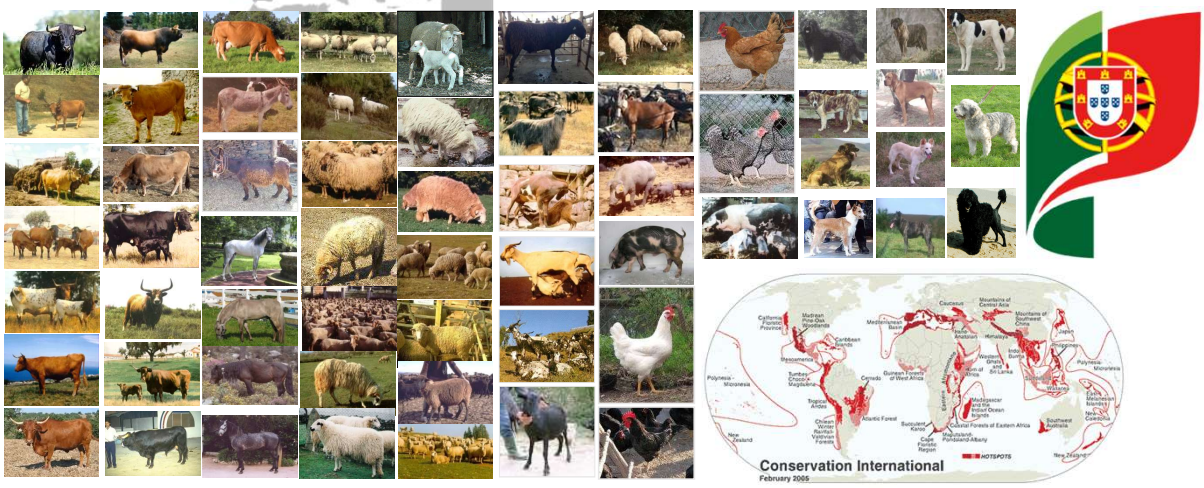
Territorial Area:
92 212 km²

52 Local Breeds

Cattle, Sheep, Goats, Pigs, Chicken, Horses, Asinine

+

11 Local Dog Breeds



Ex-situ Conservation: Cryoconservation

North

Centre

South

BPGA
BANCO PORTUGUÊS DE GERMOPLASMA ANIMAL

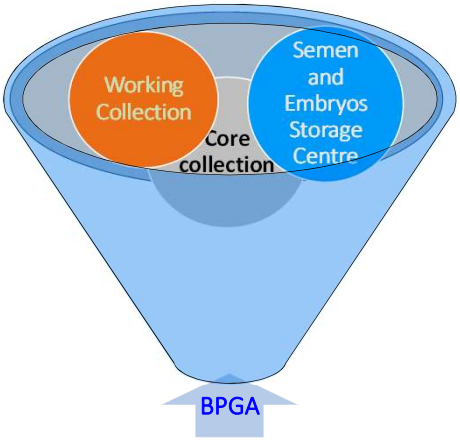
Duplicate collections

Main GeneBank

Portuguese Animal Germplasm Bank (since Sept 2010)

BPGA Committee

- 2 INIAV
- 2 DGAV
- 9 Representative Stakeholders including breeders associations



Semen
Embryos
Oocytes
DNA
Ovarian tissue

INIAV and DGAV provide the facilities, qualified technical staff and running costs (budget of the Ministry of Agriculture and Fisheries)

Portuguese GENEBANK

Species	Breed	Semen		Embryos	
		Males	Dosis (n)	Females	n
Cattle	Alentejana	66	32282	53	55
	Algarvia	7	4472		
	Arouquesa	30	16147		
	Barrosã	105	20763		
	Brava Lide	28	5987		
	Cachena	25	5554		
	Garvonesa	20	6109		
	Jarmelista	2	700		
	Marinhoa	32	4933		
	Maronesa	43	10706		
	Mertolenga	81	29885	2	6
	Minhota	57	10980		
	Mirandesa	39	10767		
	Preta	50	19643		
	Ramo Grande	15			
Pigs	Alentejana				
	Bisara				
	Malhado Alcobaça	1	60		
Horses/Donkeys	Burro de Miranda				
	Burro Graciosa				
	Garrano	19	576		
	Pónei Terceira				
	Soraia	5	363		
	Lusitano				

Species	Breed	Semen		Embryos	
		Males	Dosis (n)	Females	n
Sheep	Bordaleira EDM	9	1888		
	Campaniça	29	6274	15	75
	Churra Algarvia	3	656		
	Churra Campo	21	3342		
	Churra Minho	12	1024		
	Churra Badana				
	G. Bragançana Branc	15	4292		
	G. Bragançana Preta	10	2714		
	Galega Mirandesa	12	2895	6	20
	Churra T Quente				
	Merino Beira Baixa	6	1090		
	Merino Branco	31	7284		
	Merino Preto	27	6234	29	191
	Mondegueira	5	891		
	Saloia	18	4264		
	Serra da Estrela	41	6077		
Goats	Algarvia	8	1666	7	41
	Bravia	15	1689	8	17
	Charnequeira	11	2138		
	Preta Montesinho	11	1920		
	Serpentina	33	5801	16	100
	Serrana	39	8294	22	45

Ram Semen Cryopreservation in Portuguese Native Breeds: season and breed effects on Semen Quality Variation

Table 1. Ejaculate volume, spermatozoa concentration and total sperm per ejaculate in the 10 local ram breeds (n=1471) (Barbas et al., 2023)

Breed	SPZ traits*		
	Volume per ejaculate (mL)	SPZ concentration (10^6/mL)	Total per ejaculate (10^6)
Serra da Estrela	0.84±0.02^{a}	5283±66^{a,b}	4396±79^{a}
Churro Galego Mirandês	0.72±0.03^{a,b,c}	5916±79^{a}	4319±93^{a}
Saloio	0.88±0.03^{a}	4889±79^{a,b}	4315±93^{a}
Bordaleiro Entre Douro e Minho	0.54±0.02^{c}	5.303±83^{a,b}	2881±100^{a,b}
Churro Galego Bragançano	0.81±0.03^{a,b}	3879±98^{b}	3248 ±115^{a,b}
Churro do Campo	0.52±0.05^{b,c}	3293±136^{b}	1764±169^{b}
Merino Beira Baixa	0.58±0.04^{b,c}	5068±125^{a,b}	2990±149^{a,b}
Mondegueira	0.83±0.04^{a,b}	3959±125^{a,b}	3291±147^{a,b}
Churro Algarvio	0.87±0.05^{a,b}	4011±159^{a,b}	3581±188^{a,b}
Merino Branco	0.85±0.06^{a,b,c}	5423 ±197^{a,b}	4718±234^{a,b}

The ejaculate mean volume and SPZ concentration were affected by breed but not by season

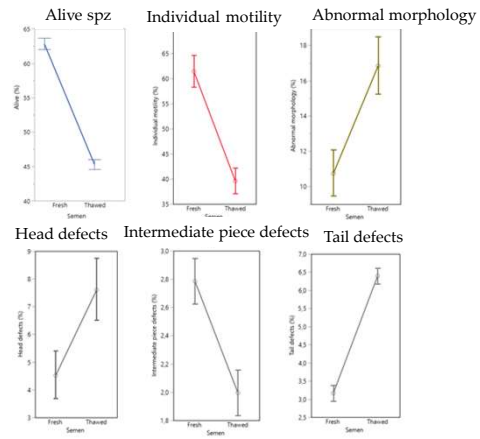
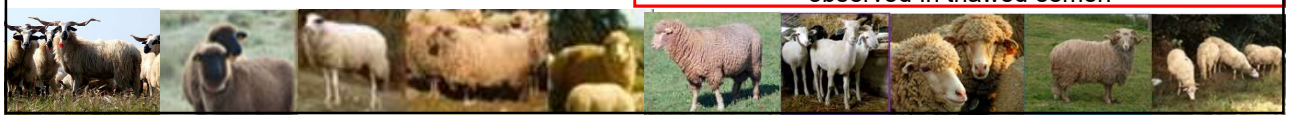


Figure 1. Differences (P<0.001) between fresh and thawed semen regarding 10 Portuguese local ram breeds.

A significant decrease of SPZ viability and individual motility, and an increase of abnormal morphology was observed in thawed semen



Ram Semen Cryopreservation in Portuguese Native Breeds: season and breed effects on Semen Quality Variation

Table 2. Effects of breed, season and semen cryopreservation on reproductive SPZ traits (Barbas et al., 2023)

Spermatozoa (%)	Breed	Season	Thawing	Breed x Season	Breed x Thawing	Season x Thawing	Breed x Season x Thawing
Alive	**	NS	***	NS	***	NS	NS
Individual motility	*	NS	***	*	NS	NS	**
Abnormal morphology	**	NS	***	**	***	*	NS
Head defects	***	NS	***	***	***	NS	NS
Intermediate piece defects	*	***	***	***	*	*	NS
Tail defects	NS	NS	***	NS	**	NS	NS

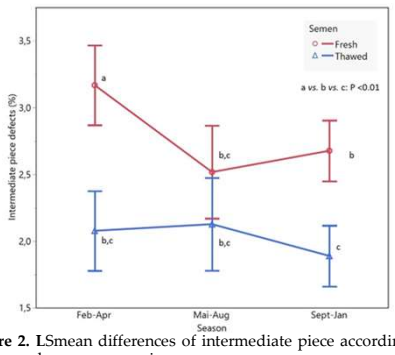


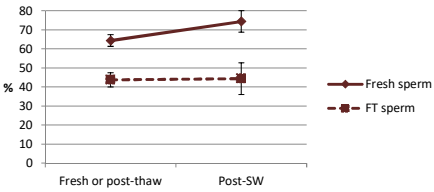
Figure 2. LSmean differences of intermediate piece according the season and semen processing.

Effects of semen processing and breed, but not of season (except IP defects), were observed on SPZ traits. Breed x season interactions influenced individual motility and abnormal morphology.

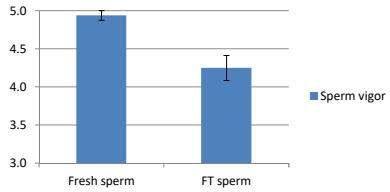
Overall, Portuguese local rams have low seasonality, and can be employed in natural mating as well as semen donors for cryopreservation and assisted reproductive biotechnologies during whole the year

FRESH vs. CRYOPRESERVED SEMEN

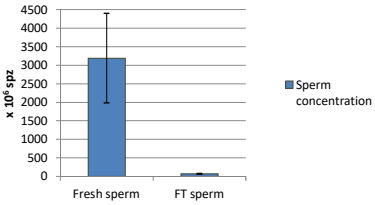
Individual motility (p≤0.02)



Post-swim-up sperm vigor (p≤0.002)



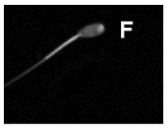
Post-swim-up sperm concentration (p=0.02)

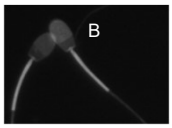


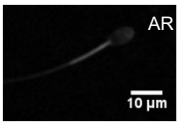
Sperm pre- and post-swim-up IM and vigor are higher in fresh sperm compared to frozen thawed (FT). Post-swim-up sperm concentration is higher in fresh sperm

Ferreira et al. 2014

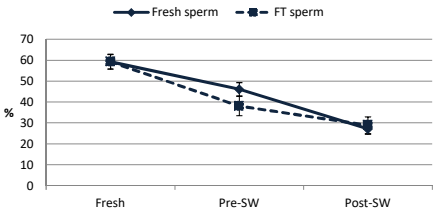
FRESH vs. CRYOPRESERVED SEMEN



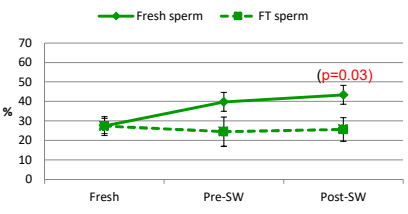




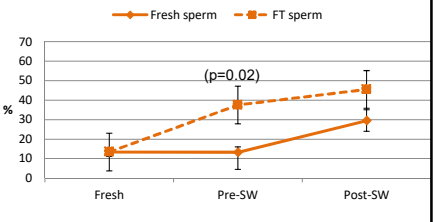
Evolution of non-capacitated F sperm



Evolution of capacitated B sperm



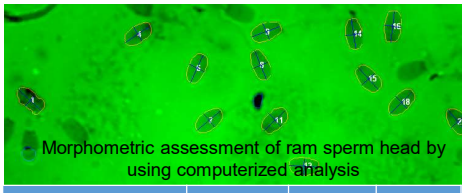
Evolution of acrosome reacted AR sperm



In post-swim-up sperm, B pattern (capacitated with intact acrosome) was higher in fresh semen. In pre-swim-up sperm, AR pattern (acrosome reacted) was higher in FT semen.

Ferreira et al. 2014

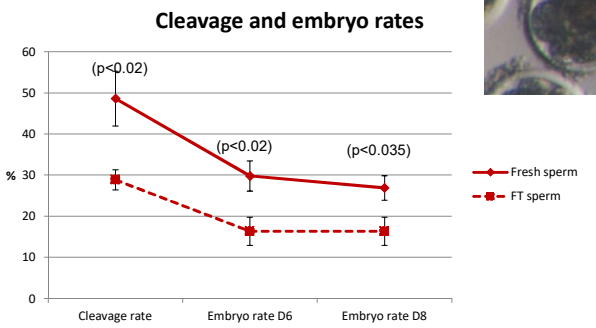
FRESH vs. CRYOPRESERVED SEMEN



- Morphometric assessment of ram sperm head by using computerized analysis

SPERM	FRESH	FT	p
HEAD AREA	25.12	24.29	0.001
HEAD PERIMETER	19.69	19.58	0.001
HEAD ELLIPTICITY	1.86	1.96	0.001
HEAD RUGOSITY	0.81	0.79	0.001

Prion protein 2 (dublet) gene (*PRND*): role in ovine semen capacitation, cryopreservation and fertility



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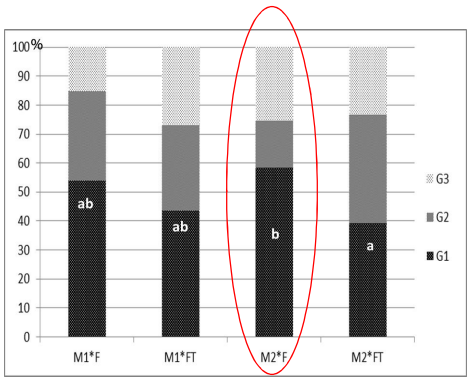
FRESH vs. CRYOPRESERVED SEMEN



Distribution of morphological quality of sheep *in vitro* produced embryo (G1, G2, G3)

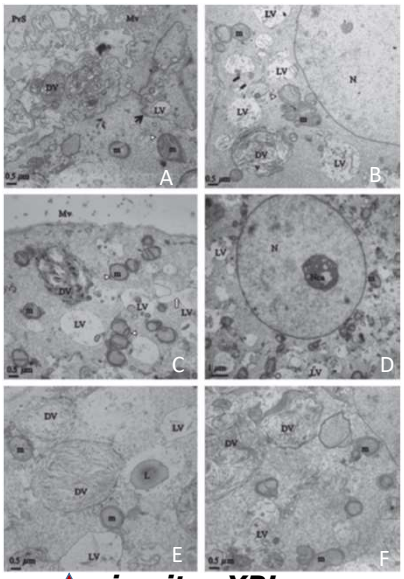
Small Ruminant Research

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

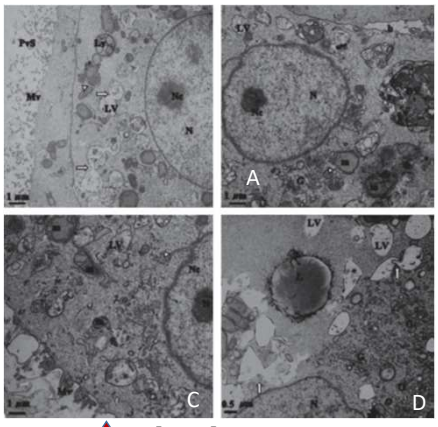
Fresh semen improved blastocyst quality, grade 1 embryos (p=0.01)

F	**52.5±4.8%**
FT	**39.0±4.4%**

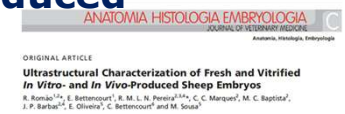
Electronic Microscopy comparing in vivo and in vitro produced sheep embryos



▲ *in vitro YBL*



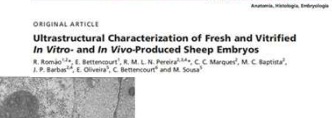
▲ *in vivo YBL*



Outer (in vitro A, C, E, in vivo A,C) and inner cells (in vitro B, D, F; in vivo B,D). 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) and nucleolus (Nc).

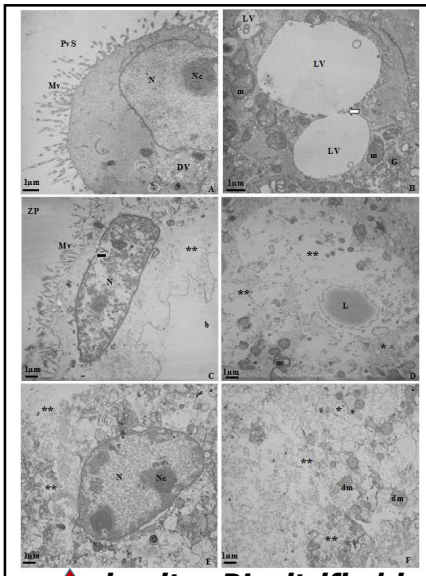
IVP fresh embryos showed a reduced number of microvilli and mitochondria, and a lower number of mature mitochondria. Also a larger number of vesicles, with light and dense content and LD were identified

Electronic Microscopy Sheep Embryos

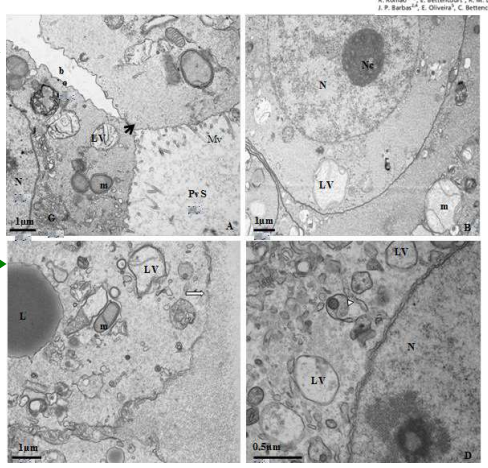


◀ Grade 1 ▶

◀ Grade 2 ▶



▲ *in vitro BL vitrified in OPS*

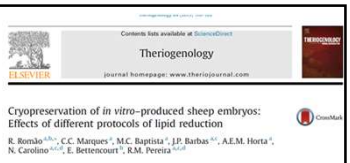


▲ *in vivo BL vitrified in OPS*

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 (*), and cell lysis (**)

Major changes were observed mainly in IVP embryos with small changes in grade 2 (fair) and high changes in grade 3 (bad), including disruption of cellular membranes and poor intracellular preservation, with loss of microvilli and the presence of cellular debris

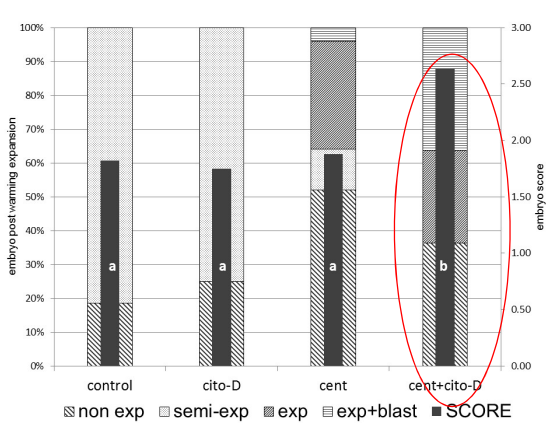
Ultracentrifugation and Cytochalasin D



In vitro sheep embryo production → Tratament → **Vitrification / Warming**

Treatment	n	Post warming integrity (%)
Control	27	**74.6 ± 9.2^{a}**
Ultracentrigutation (15000 x g)	25	**50.6 ± 10.3^{b}**
Cytochalasin D (5µgmL^{-1})	24	**92.3 ± 9.7^{a}**
Ultracentrifug + Cytochalasin D	22	**90.5 ± 11.2^{a}**

Ultracentrifugation, Cytochalasin D e CLA



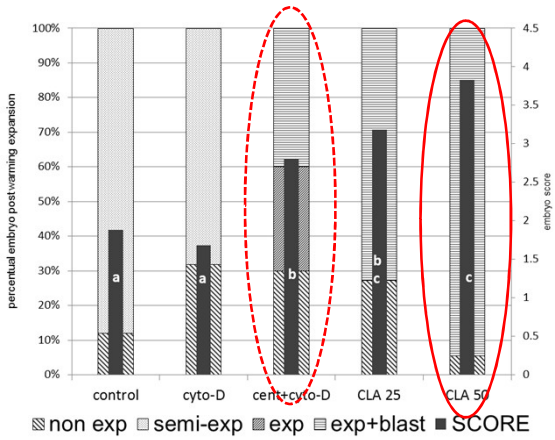


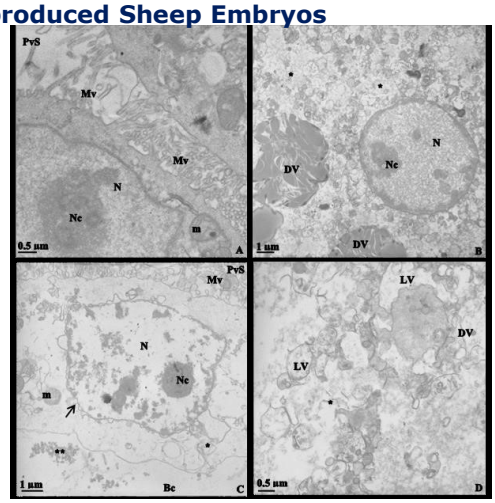
FIG. 1 Post warming evaluation of vitrified sheep embryos (experiment 1 and 2).
SCORES: non exp=1; semi-exp=2; exp=3; exp+ blast=4

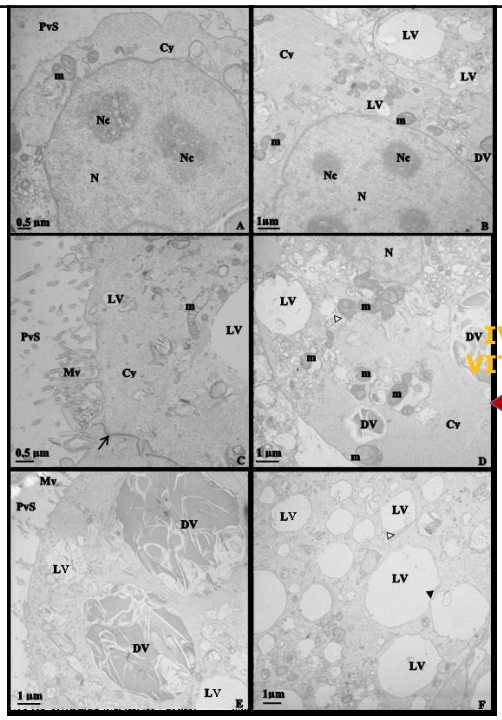
EXP 1 Best re-expansion scores were obtained in cent+cyto-D embryos ($p<0.05$)

EXP 2 After warming superior expansion scores were achieved in CLA groups ($p<0.02$)

Electronic Microscopy of in vitro produced Sheep Embryos

IVP VITRIFIED BL





IVP CLA VITRIFIED BL

CLA IVP embryos presented more abundant mitochondria showing transverse cristae compared to control, where severe signs of cell lysis were seen with disrupted membranes and the presence of more cell debris

BPGA : Bank of the Portuguese Animal Germplasm

BPGA
BANCO PORTUGUÊS DE GERMOPLASMA ANIMAL

Thank you. Linde Rombaut will proceed

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Instituto Nacional de Investigação Agrária e Veterinária, I.P.