IN SITU CONSERVATION MEETING
Toledo, May 22nd 2023

What´s new in the production and cryoconservation of porcine embryos?

Cristina Cuello ccuello@um.es
Research group ”Animal Reproduction”
Veterinary Faculty, University of Murcia, Spain

NON-SURGICAL ET

Martínez et al., 2019. Reprod Dom Anim 54(4):4-13

VITRIFICATION

Cuello et al., 2021. Front Vet Sci 8:771996
Martínez et al., 2019. Reprod Dom Anim 54(4):4-13
Martínez et al., 2016. Theriogenology 85:152-61

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Martínez et al., 2019. Reprod Dom Anim 54(4):4-13
Martínez et al., 2016. Theriogenology 85:152-61

Ineffective
poor **outcome**

NON-SURGICAL ET

Martínez et al., 2019. Reprod Dom Anim 54(4):4-13

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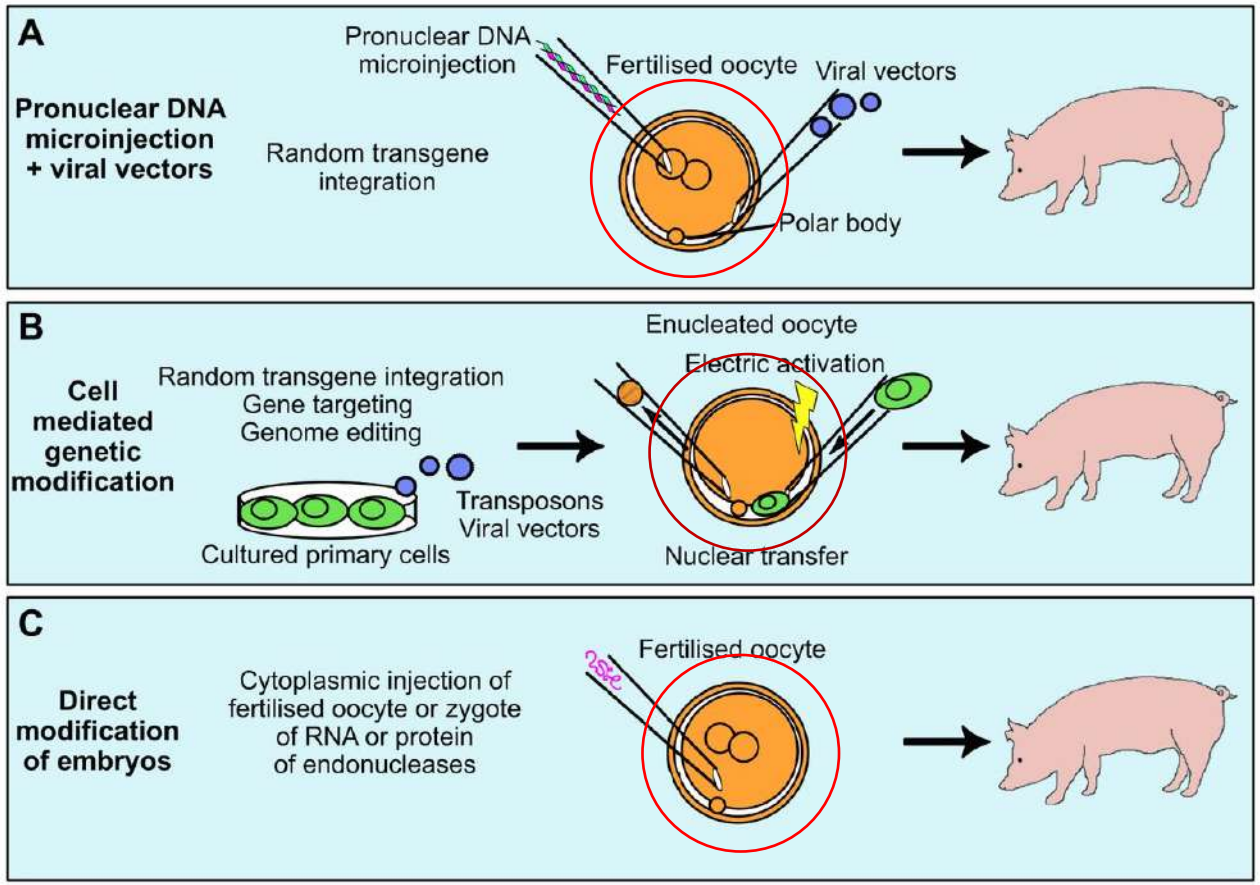
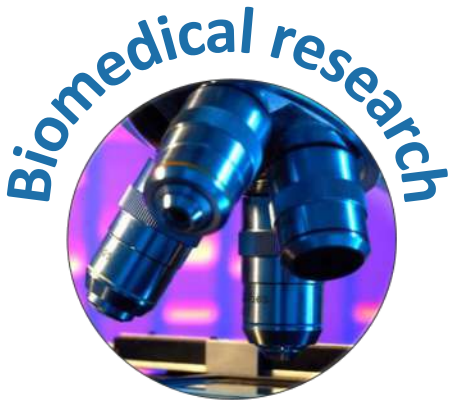
Martínez et al., 2016. Theriogenology 85:152-61

Biomedical research

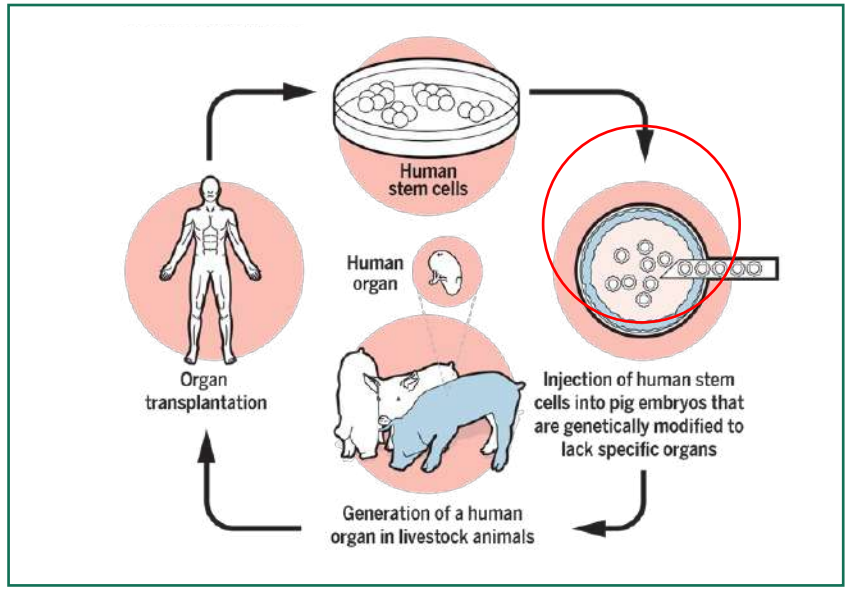
Pig models for humans...

Methods used to generate genetically modified pigs:





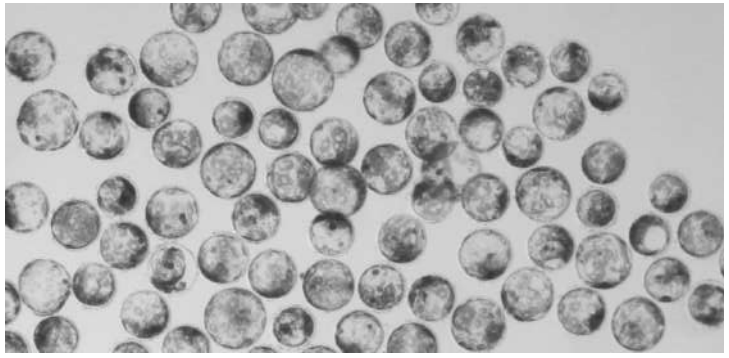
Producing human organs in pigs: Blastocyst complementation with hiPSCs



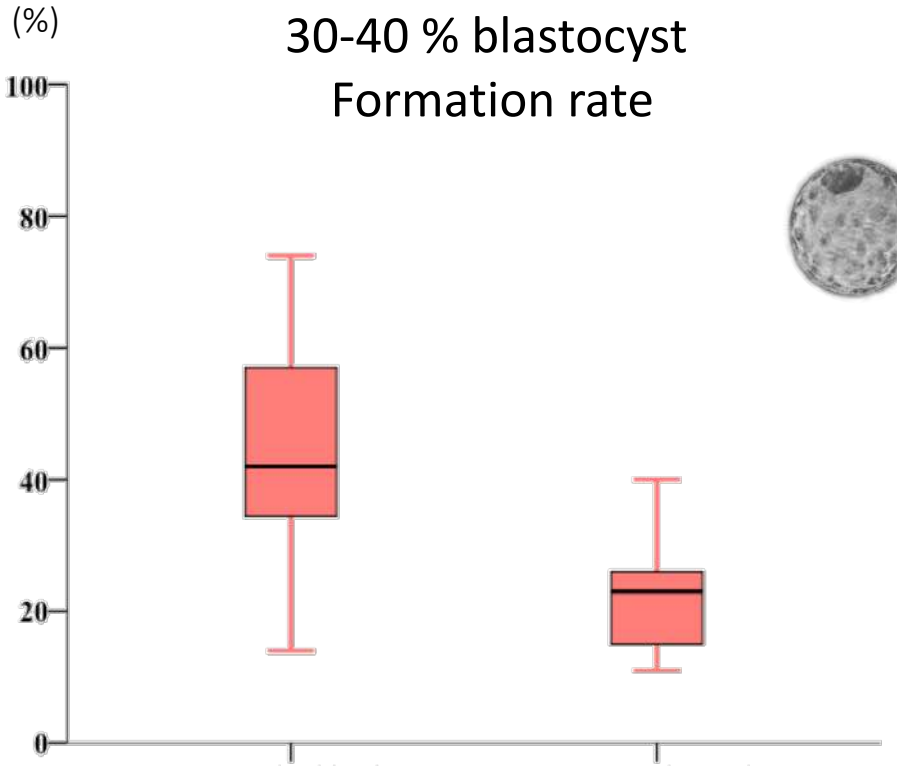
In vitro embryo production

1st Piglets born after transfer of IVP porcine embryos

(Mattioli et al., 1989. Theriogenology 31(6):1201-7)



30-40 % blastocyst Formation rate



Martinez et al., 2019. Reprod Dom Anim. 54(4):4-13.
Gil et AL., 2010. Reprod Dom Anim. 45(2):40-8.

Low efficiency because of…

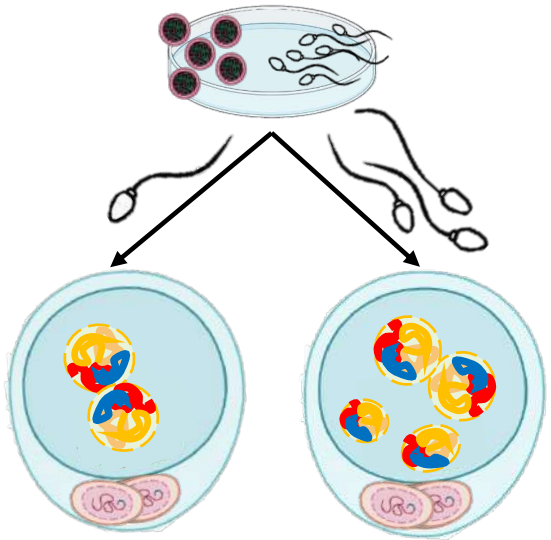
1. High polyspermy

***In vivo* <5%**

Hunter and Léglise, 1971. J Reprod Fertil 24(2):233-46

***In vitro* 60-70%**

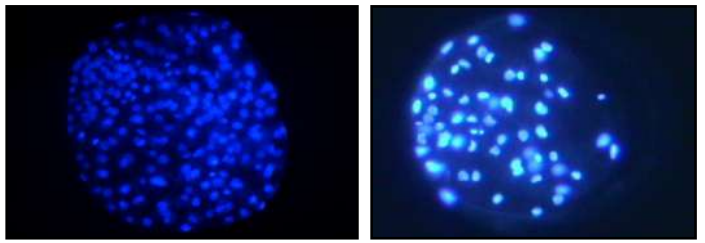
Grupen, 2014. Theriogenology 81(1):24-37



↓ Low embryo development

↑ High embryo mortality and pregnancy loss

2. Suboptimal culture conditions



In vivo *In vitro*

↓ Low embryo quality

One of the lines of research in our laboratory seeks to improve the efficiency of *in vitro* production of pig embryos

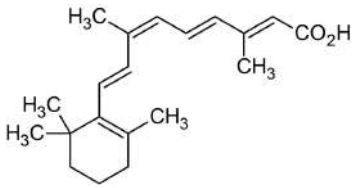
Some specific objectives:

- **Objective 1:** To improve the IVM of oocytes and IVC conditions by the addition of antioxidants.
- **Objective 2:** Reduce polyspermy in porcine IVF: sperm to oocyte ratio, co-incubation time, additives and high dilution of spermatozoa.
- **Objective 3:** Development of chemically defined conditions for IVC
- **Objective 4:** Effects of mineral oil coverage

- **Objective 1:** To improve the IVM of oocytes and the IVC conditions by the addition of **antioxidants.**

The importance of oxidative stress in *in vitro* culture conditions

(Guerin et al., 2001; Takahashi, 2012)



9-cis retinoic acid (5 nM)

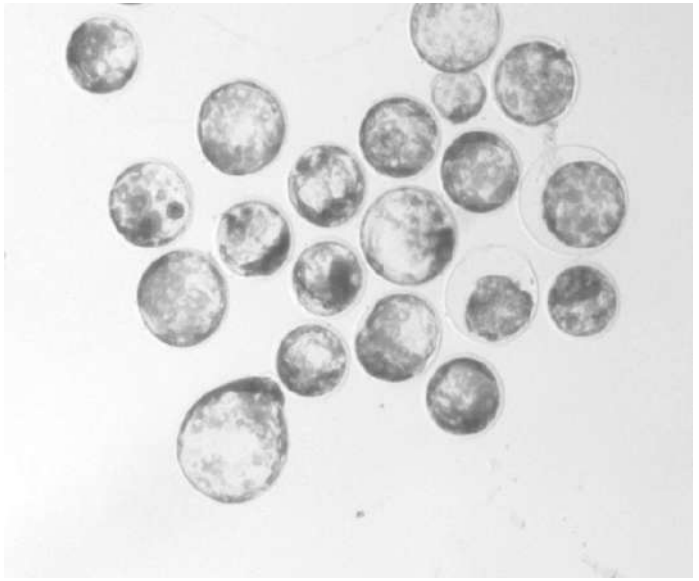
Almiñana et al. 2008. Reprod Fertil Dev 20: 483-489

MEM Vitamin Solution (0.05%)

D-Biotin, choline chloride, folic Acid, myo-Inositol, niacinamide, p-Amino Benzoic Acid, D-Pantothenic Acid, pyridoxal•HCl, pyridoxine•HCl, riboflavin, thiamine•HCl, vitamin B-12

Cuello et al, 2013. Anim Reprod Sci. 136: 296-302

IVM



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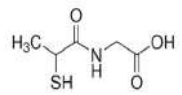


N-(2-mercaptopropionyl) glycine (NMPG; 10 µM)

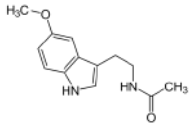
IVC

Increased blastocysts formation

Decreased intracellular ROS levels in blastocysts

Reduced expression of oxidative stress related genes

Cambra et al., 2020. Sci Rep (10):18632



Melatonin (1 nM)

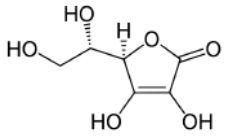
IVC

Improve blastocysts formation

Accelerated embryonic development kinetic

Improve embryo quality (decreased ROS levels and DNA damage, increased GSH and ICM cells)

Martinez et al., 2022. Antioxidants 11, 1177



Ascorbic Acid (50 µg/mL)

IVM, IVF, IVC

Improved vitrification ability of IVP porcine blastocysts

Decreased intracellular ROS levels in blastocysts

Nohalez et al., 2018. Theriogenology 113: 113-119

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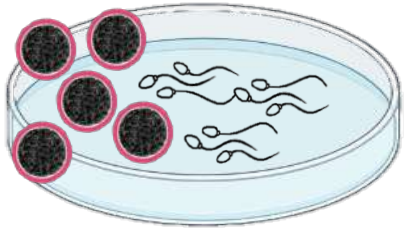
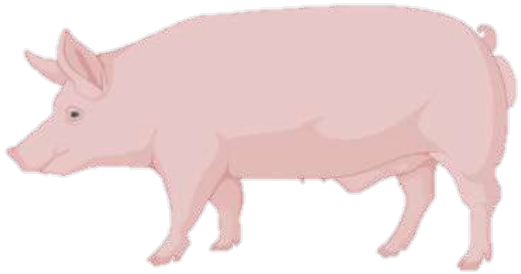


Sperm:oocyte ratio 2000, 1500, 1000 and 500.

Con-incubation time: 2min, 10 min, 6 h.

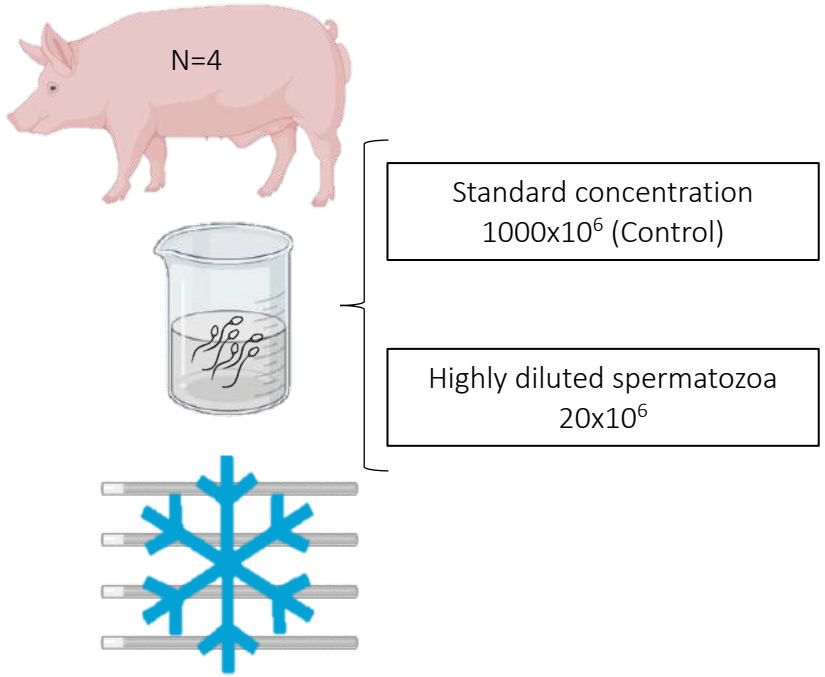
Aditives: Caffeine and hyaluronic acid

Gil et al. 2004. Theriogenology 551-560; Gil et al. 2007. Theriogenology 620-626; Almiñana et al. 2008. Anim Reprod Sci 106: 393-401.

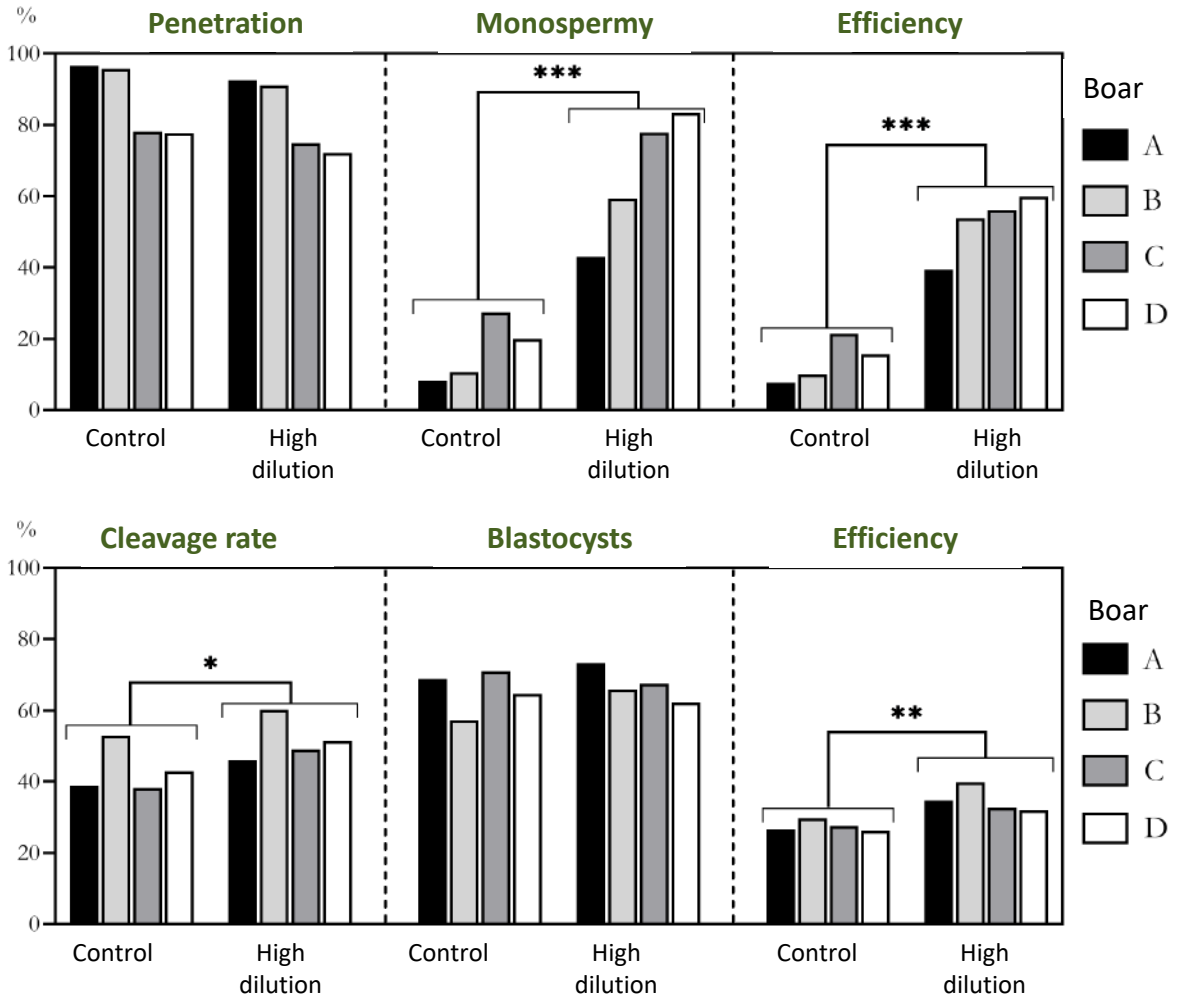


A preliminary screening for each individual boar is required to select optimal conditions for IVF

- **Objective 2:** Reduce polyspermy in porcine IVF: sperm to oocyte ratio, co-incubation time, additives and high extension of spermatozoa.



Martinez et al. 2019. Theriogenology 131:162-168



One of the lines of research in our laboratory seeks to improve the efficiency of *in vitro* production of pig embryos

Some specific objectives:

- **Objective 1:** To improve the IVM of oocytes and IVC conditions by the addition of antioxidants and XXX.
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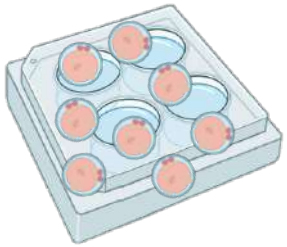
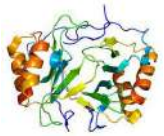


N= 3820

cytokine platelet factor 4

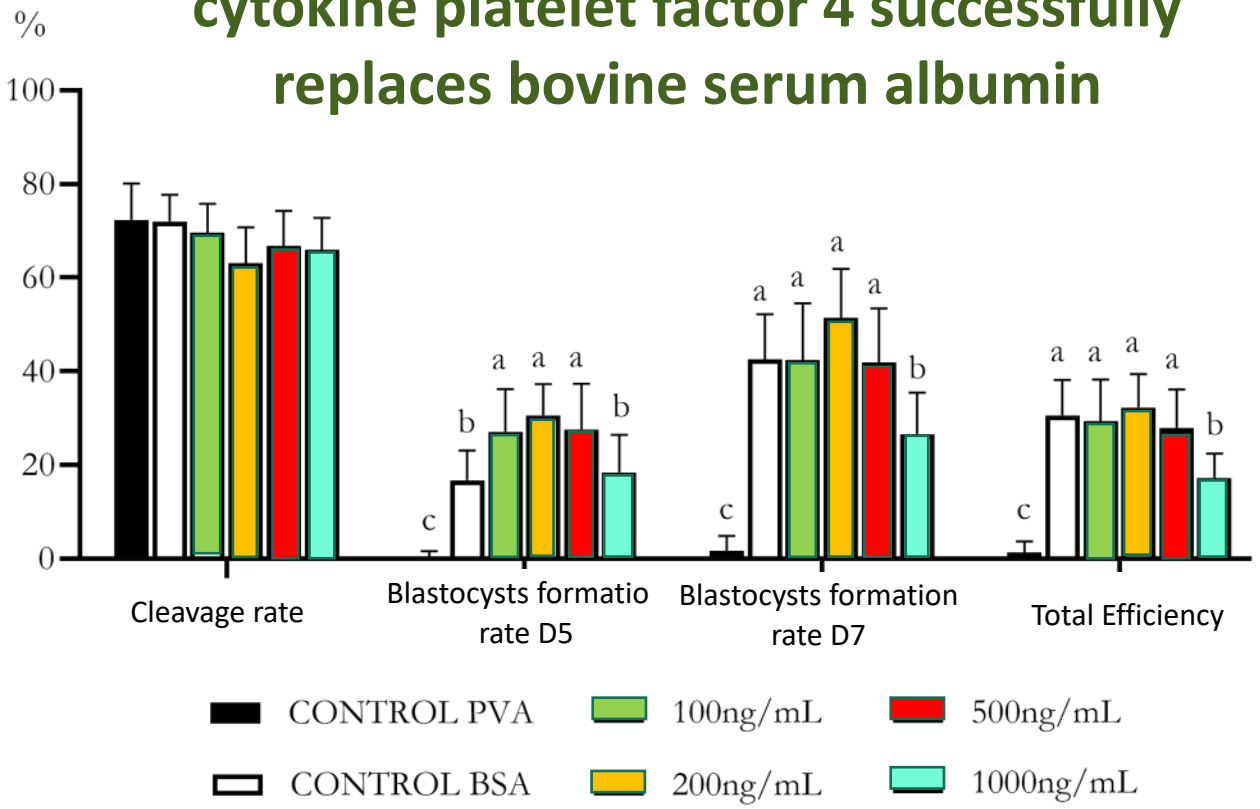


ng/mL
1000
500
200
100

IVC Base medium:
NCSU-23 supplemented with PVA

Embryo development
Cleavage
Blastocyst formation
Efficiency of CIV
Embryo quality
TCN ICM/TE
Embryo quality
Transcriptome

cytokine platelet factor 4 successfully replaces bovine serum albumin



Similar total cell number and inncer cell mass/trophectoderm cells ratio

Cambra et al. 2020. Theriogenology 148:201-207

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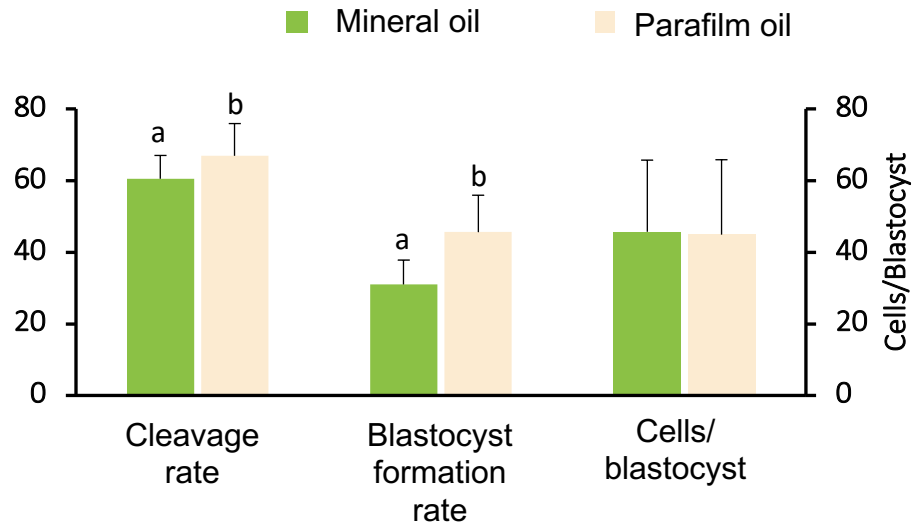
Mineral oil

VS

Parafilm oil

Embryo IVP Efficiency

	MIV	FIV	CIV
N	533	365	607



Oil

Medium

Incubation without oocytes or embryos

Peroxide values =

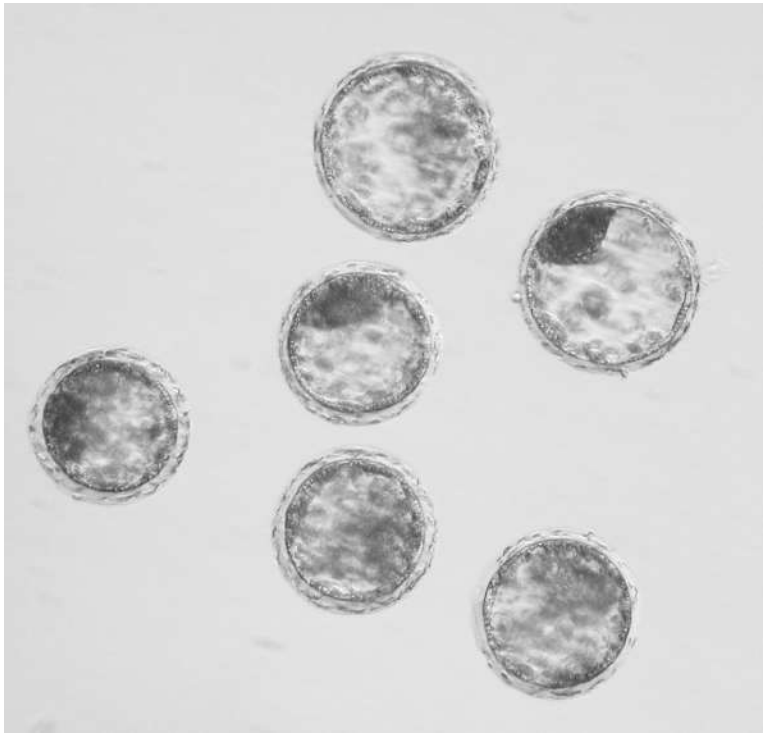
Inorganic elements =

Volatile organic compounds (VOC): Differences in both the composition of VOC and the transfer of some VOC to the culture medium

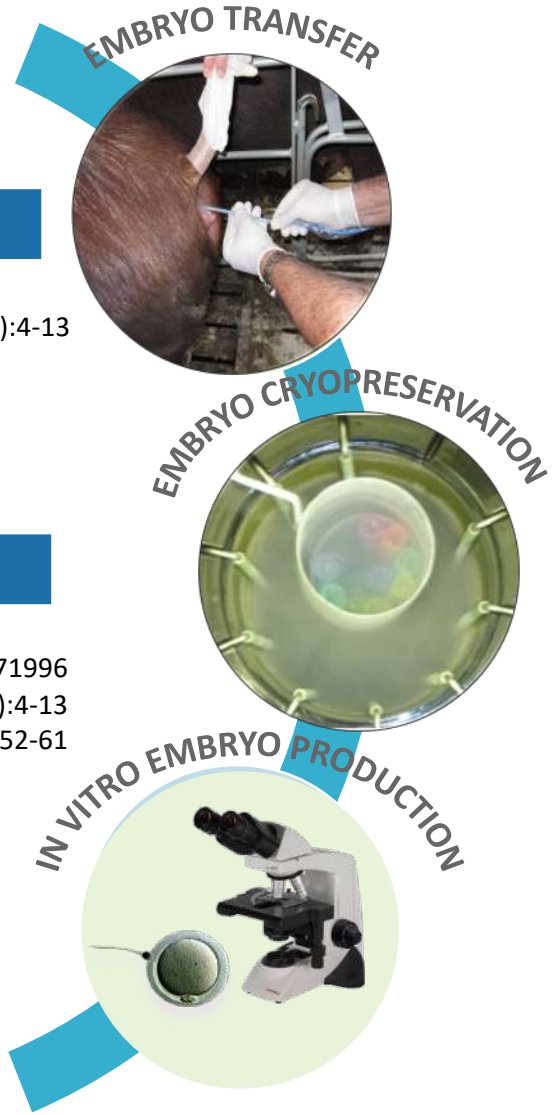
IVM medium: Days 0 and 1

IVC medium: Days 0, 2 and 5

Martinez et al., 2017. Sci Rep 7(1):10505



In vivo-produced embryos



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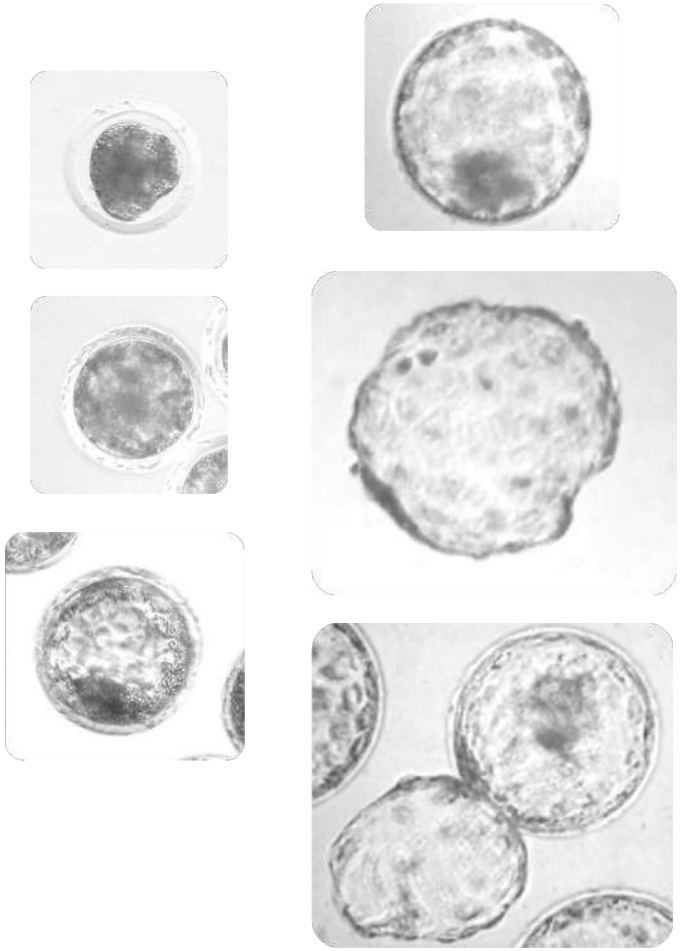
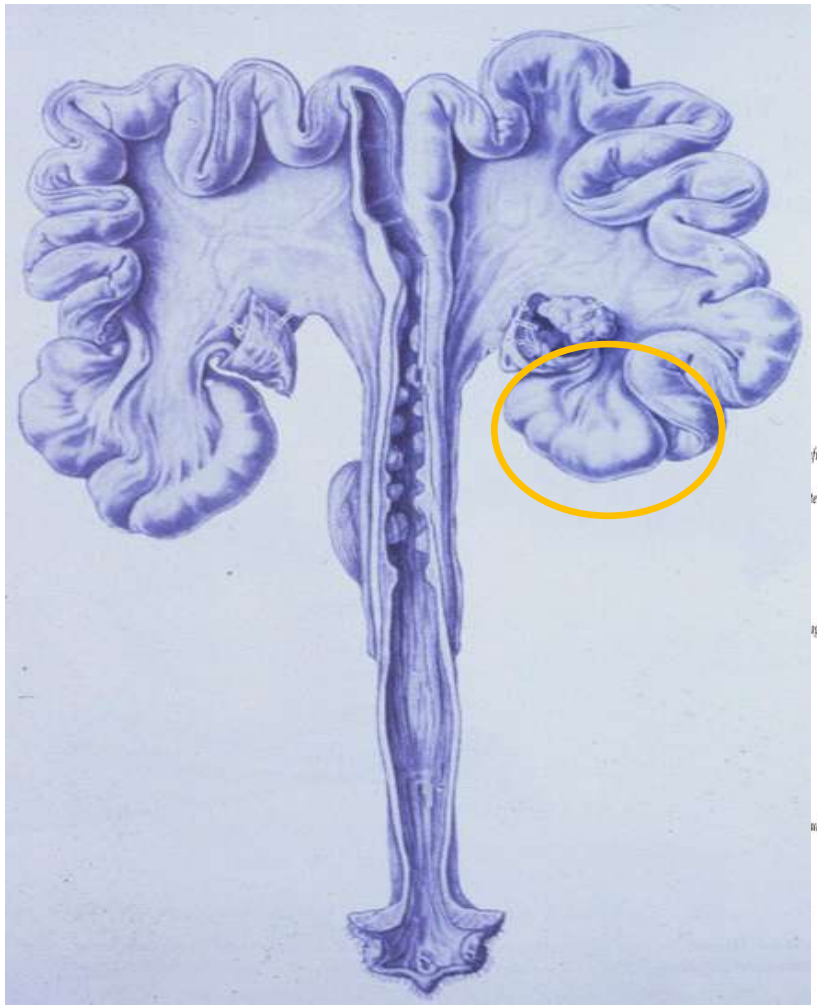
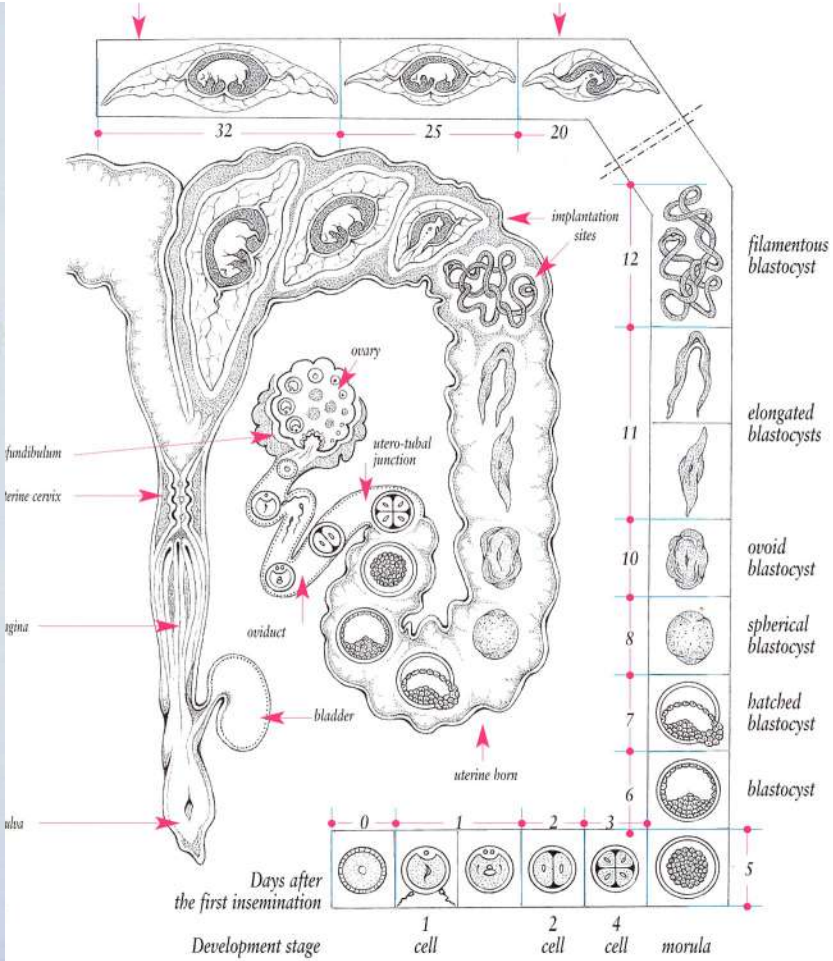
In vivo embryo production

How do we obtain pig embryos for vitrification and /or ET?







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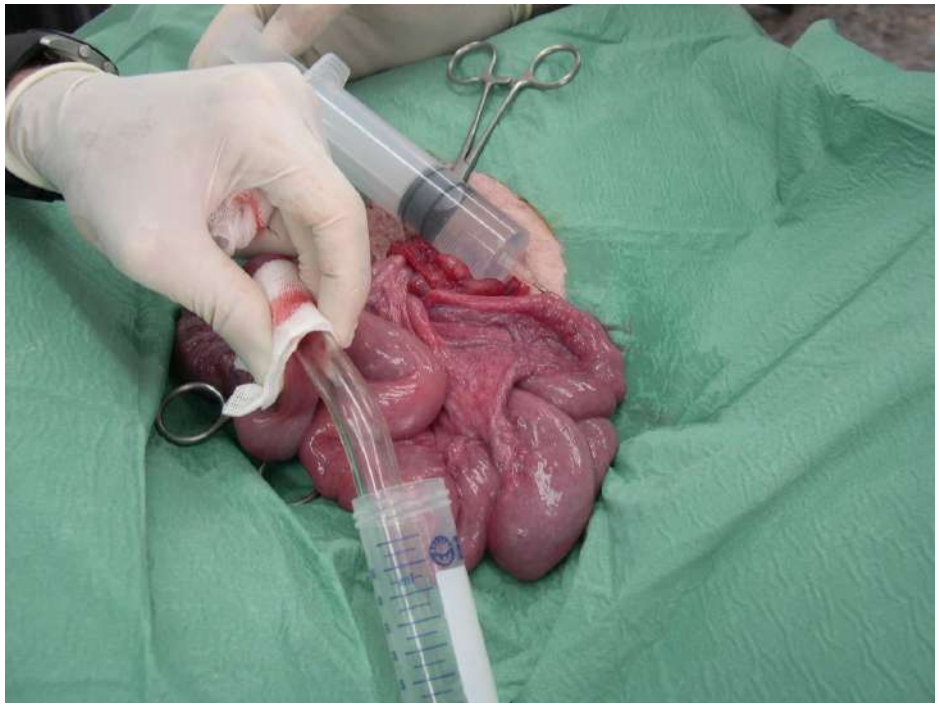
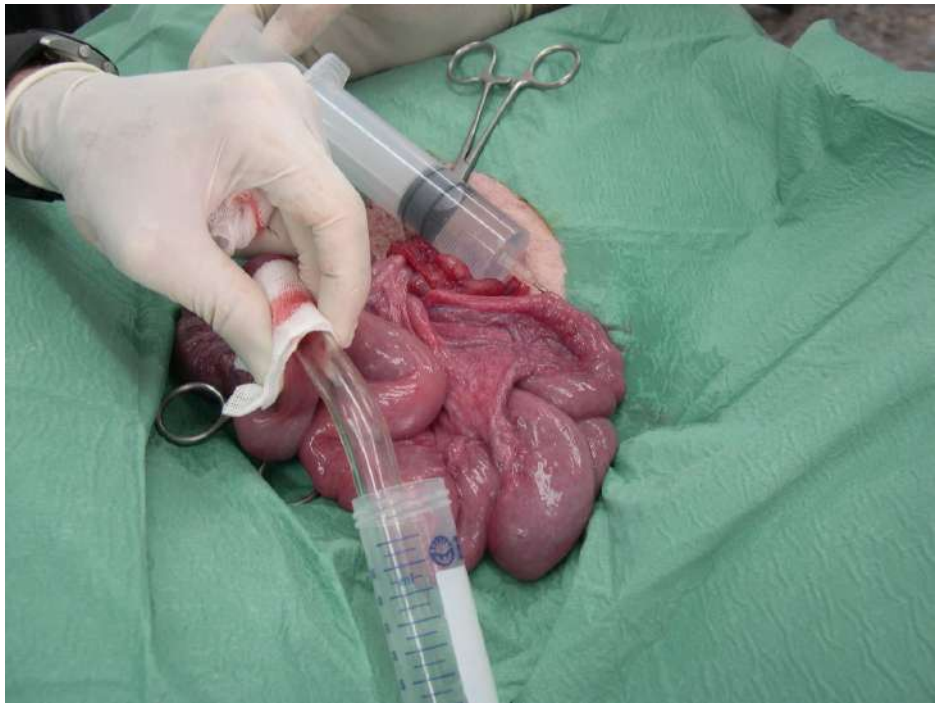
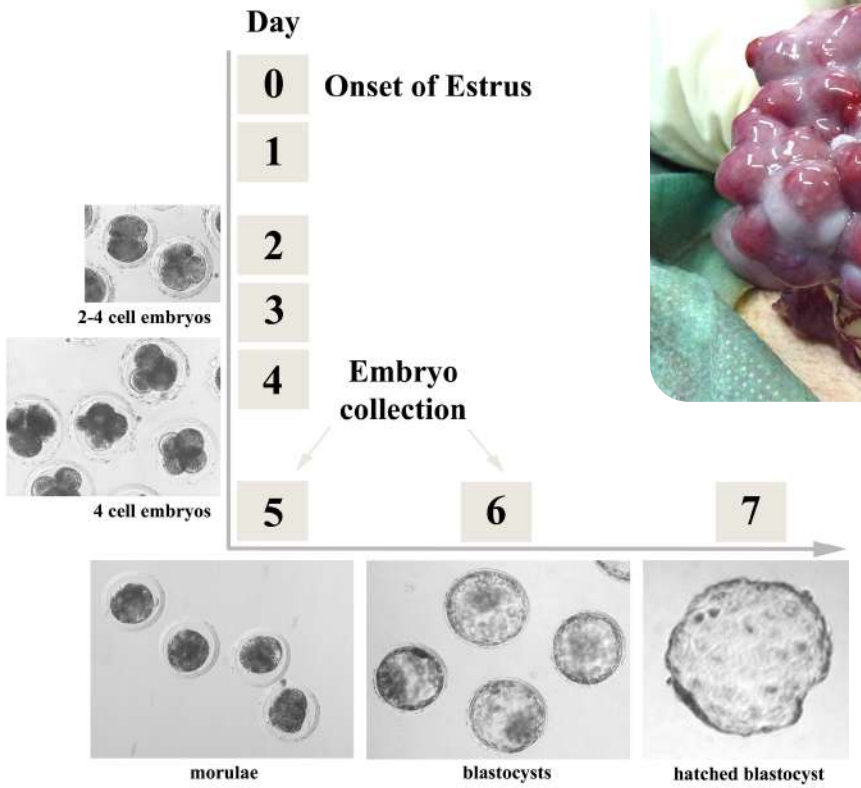
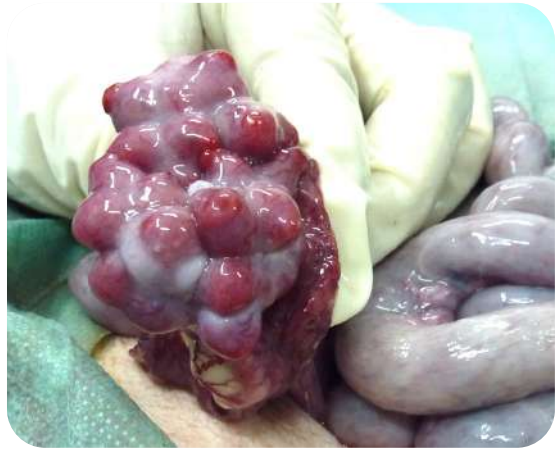


How do we obtain pig embryos for vitrification and /or ET?

When?







How do we obtain pig embryos for vitrification and /or ET?

Ovulation rate: 15-25 oocytes

No. of embryos/TE:

Surgical: 15-23

Non-surgical: 24-30

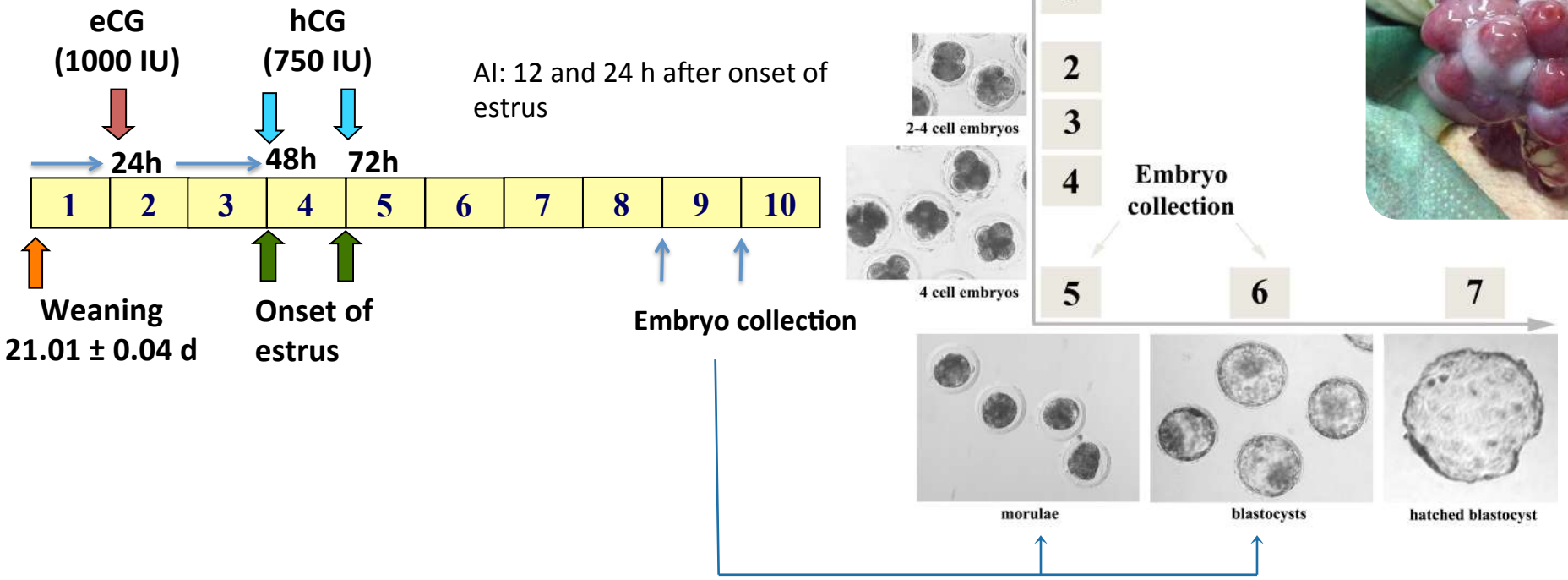
Teorical ration donor:recipient	**1:1**

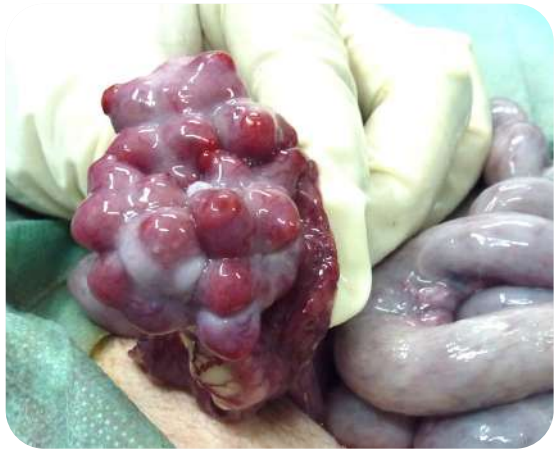
Factors affecting the number of collected embryos

- Gestation rate
- Fertilization rate
- Embryo developmental stage
- Embryo quality
- Recuperation rate

Real ratio donor:recipient	**2-2.5:1**

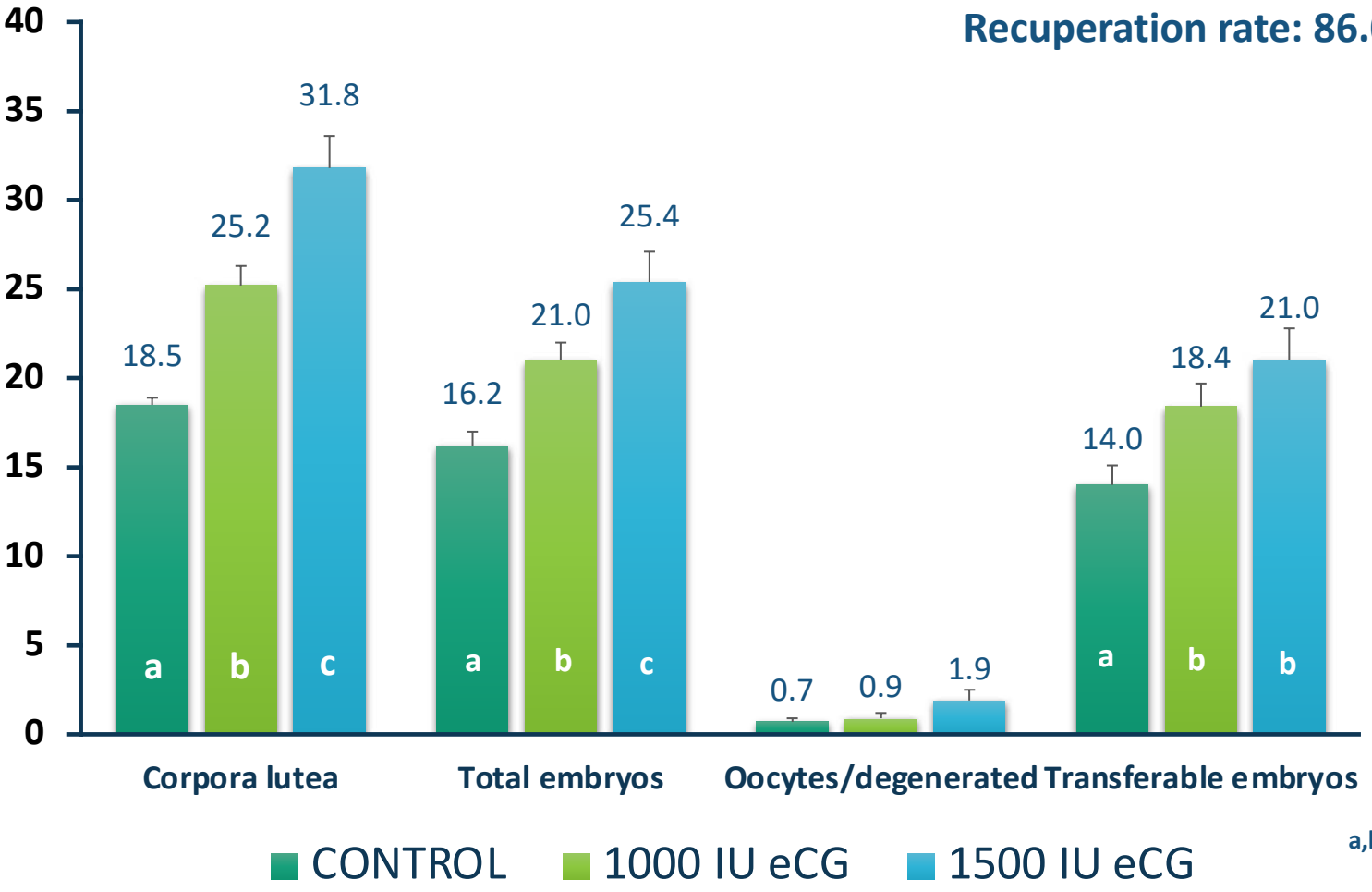
How do we obtain pig embryos for vitrification and /or ET?





Duroc (n=78)

Recuperation rate: 86.0% - 90.1%

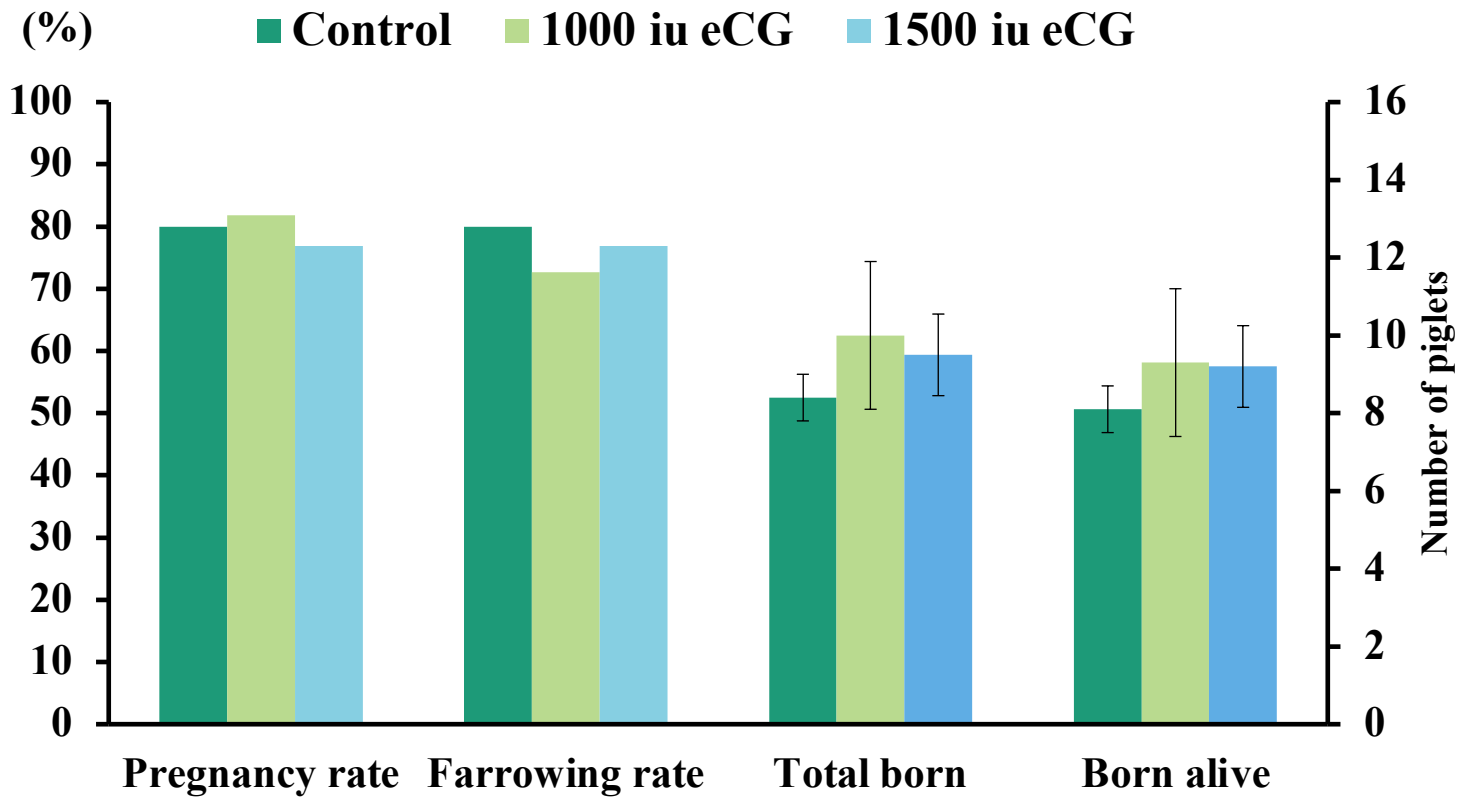


	Control	Superovulated
Donor:recipient	2.1:1	1.5:1

^{a,b,c,} $P < 0.05$

Angel et al., 2014. Theriogenology 81(6):832-9.

Reproductive parameters after transfer of recipients (n=34) with embryos (30 per recipient) collected form superovulated donors



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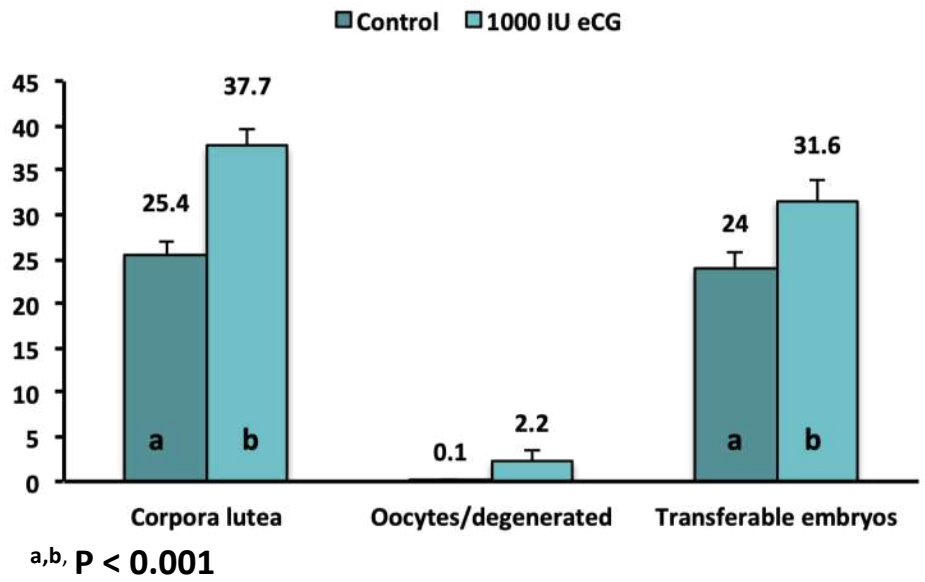
Angel et al., 2014. Theriogenology 81(6):832-9.

Landrace x Large-White (n=104)

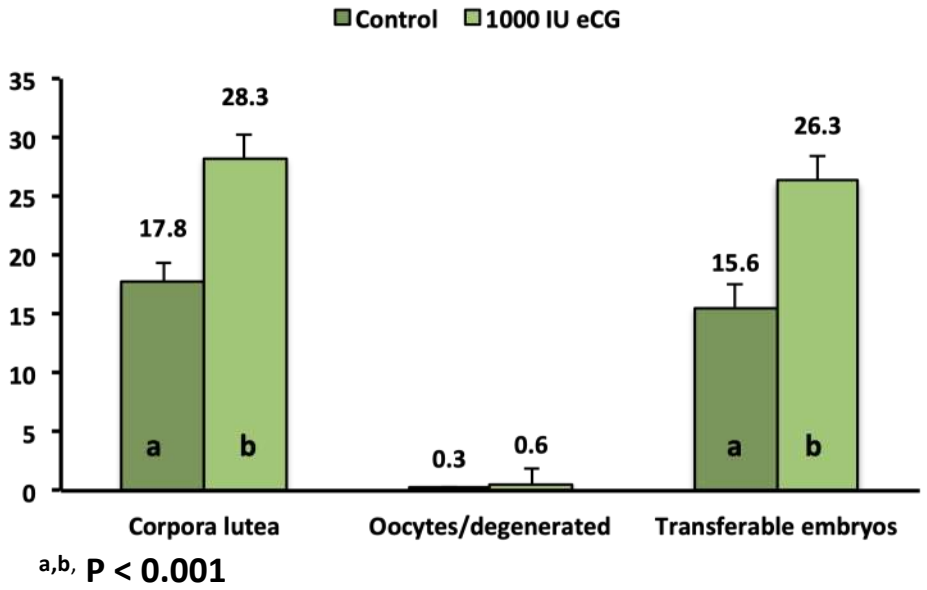


a,b, P < 0.001

Recovery rate: 85.3% - 88.7%

	Control	Superovulated
Donor:Recipient	1.2:1	0.9:1

Pietrain (n=30)

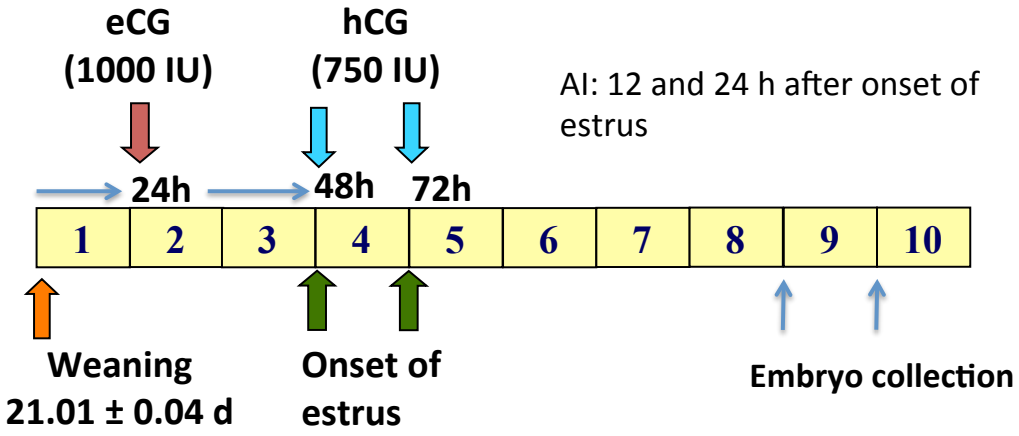


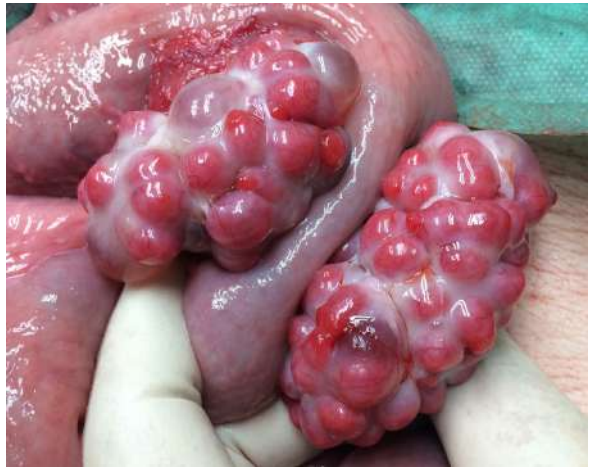
a,b, P < 0.001

Recovery rate: 90.3% - 92.8%

	Control	Superovulated
Donor:Recipient	1.9:1	1.1:1

Cuello et al., 2016. ICAR conference Tours, France



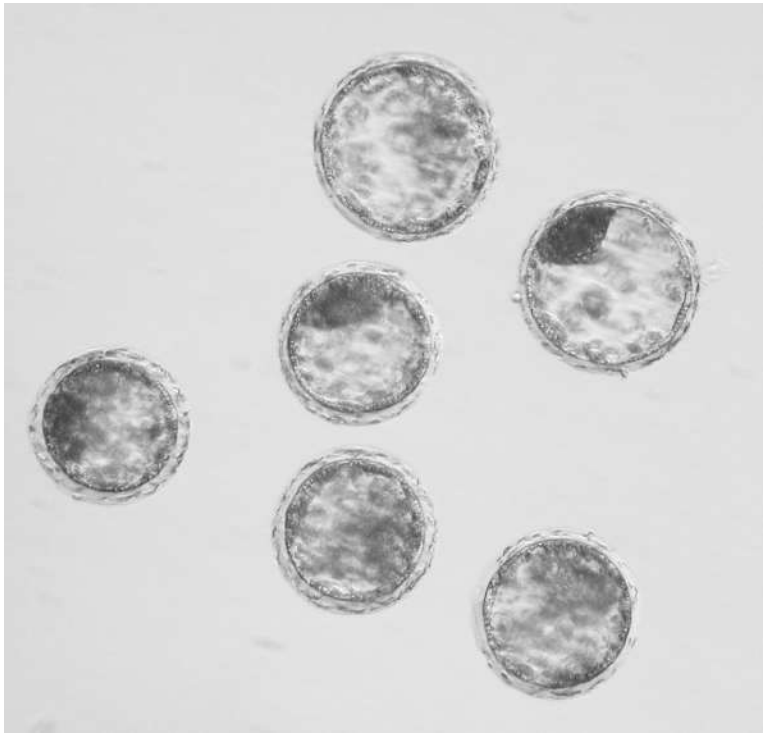


Efficiency of superovulation is NOT influenced by:

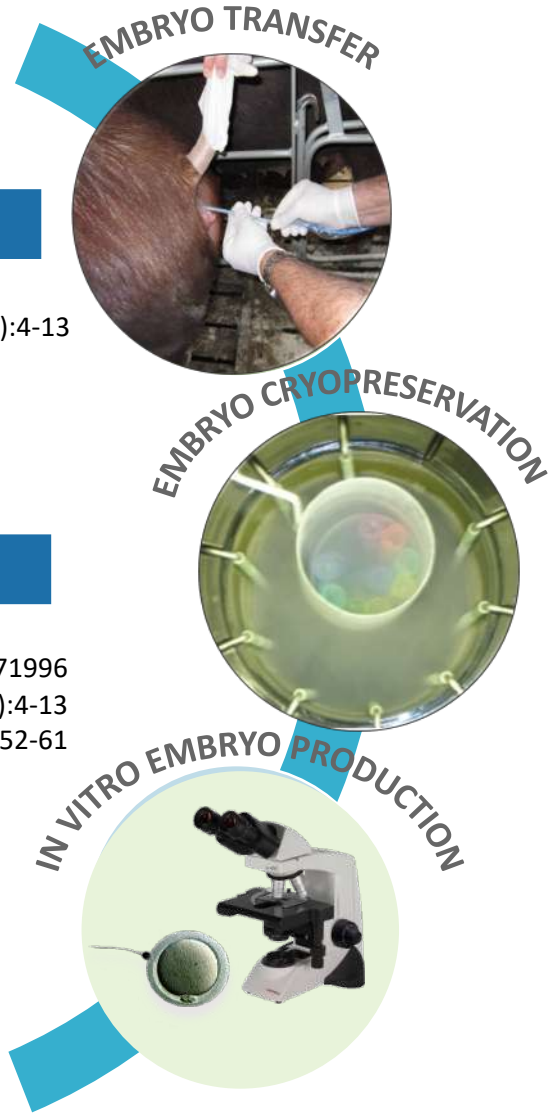
- Parity (2-7 parities)
- Season (autumn, winter, spring)
- WEI (3 to 4 days)

→ **Facilitate the use of ET programs in donor farms**

Nohalez et al., 2017. Animal 11(8):1330-35



In vivo-produced embryos



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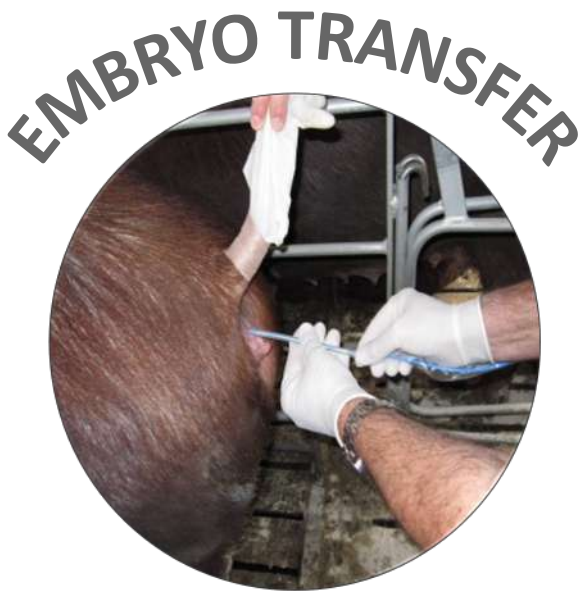
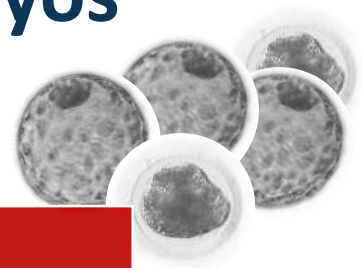
The best way to exchange genetic material ... the embryos



- Cost effective
- Minimal sanitary risk
- No animal welfare problems related to transport

The best way to exchange genetic material ... the embryos

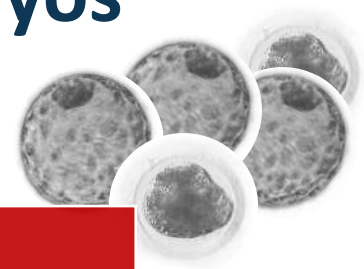


Limitations

EMBRYO TRANSFER

- Surgical embryo collection
- Surgical embryo transfer
- Difficulties associated to the embryo cryopreservation

The best way to exchange genetic material ... the embryos



Limitations

EMBRYO TRANSFER

- Surgical embryo collection
- Non-surgical embryo transfer
- Development of vitrification

Non-surgical embryo transfer

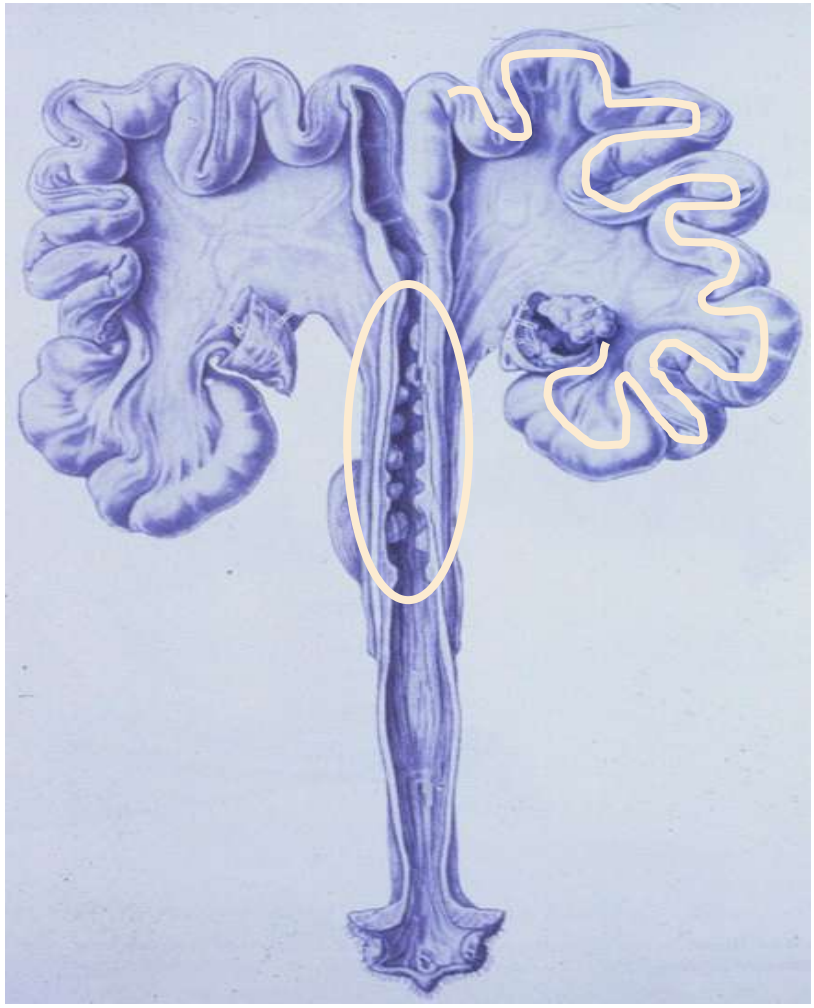
Genital tract Anatomy

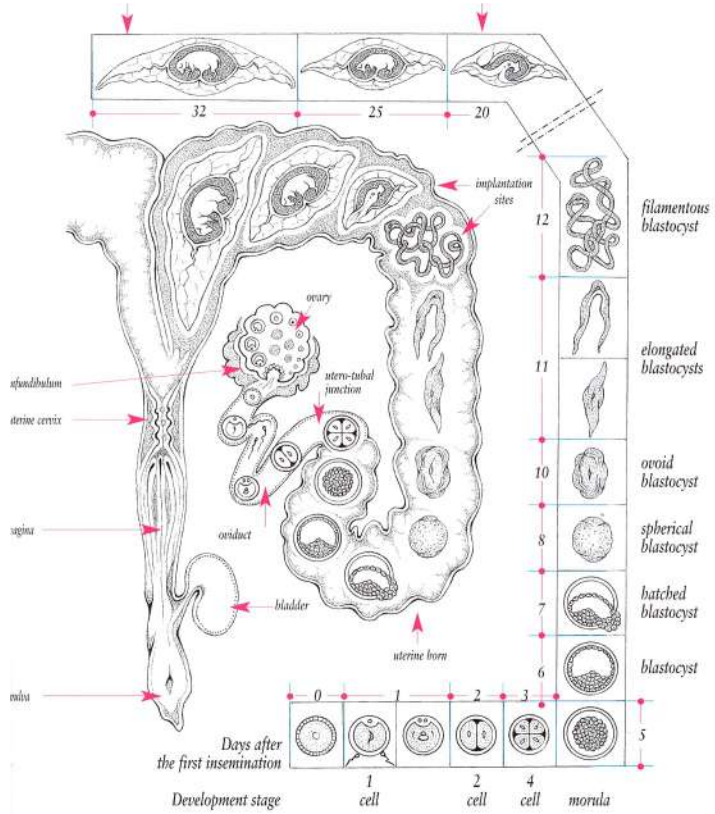
Long cervix and prominent cervical folds

Very long uterine horns

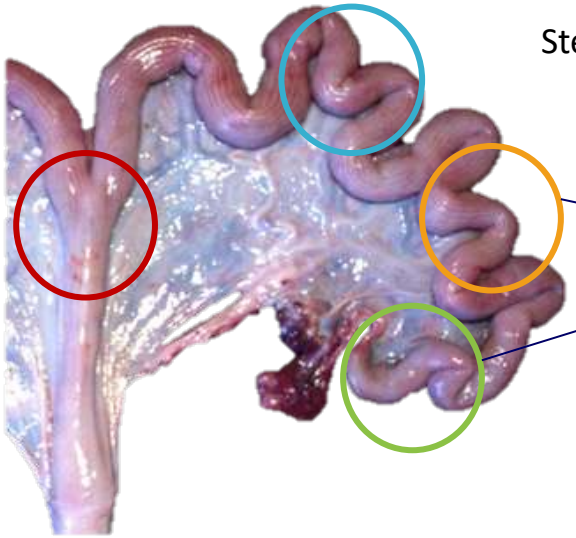
Embryo collection and transfer

Surgical procedures





SURGICAL EMBRYO TRANSFER



Stein-Stefani and Holtz, 1999. J Anim Sci 77(9):2327-9)

Gestation rate (%)	
Uterine body	12
Uterine horn (middle)	88
Uterine horn (anterior region)	81

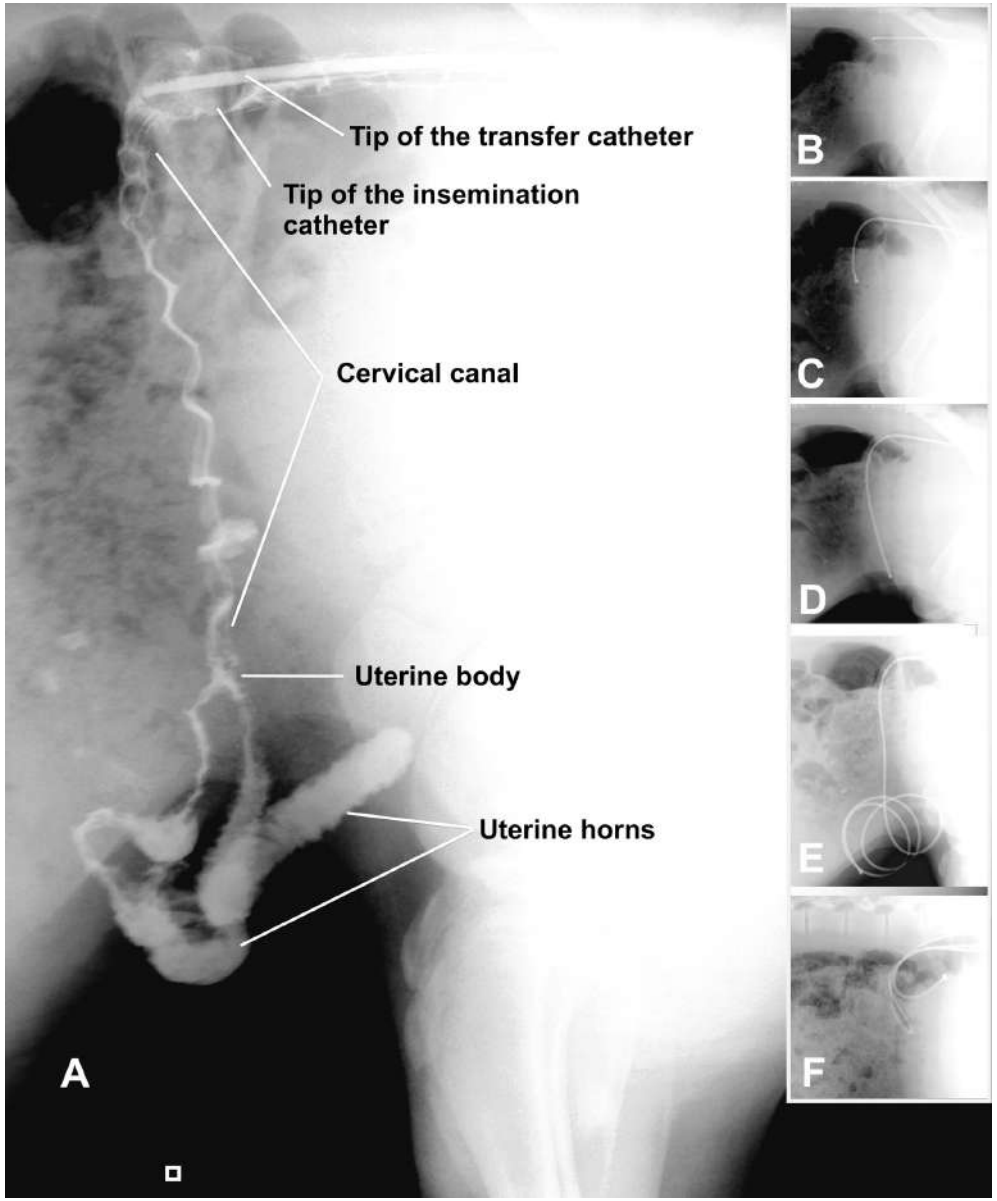
Similar results

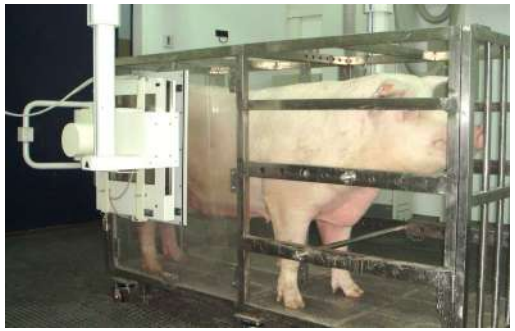
↑

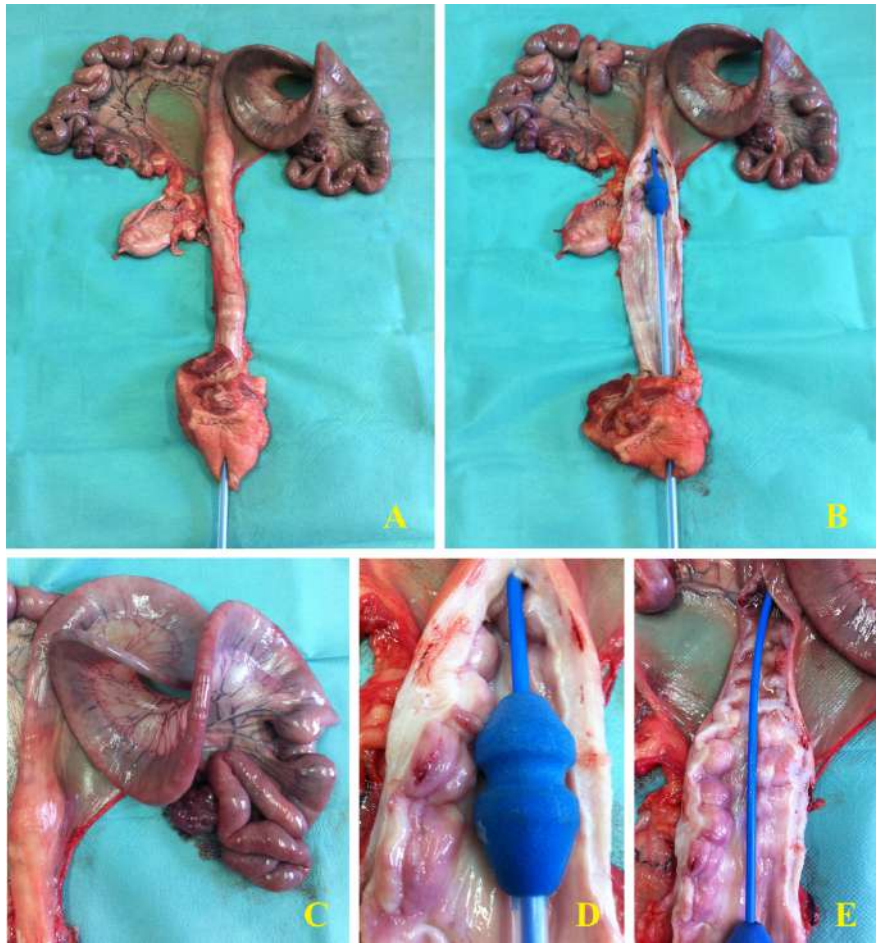
Physiological place of D5-D6 embryos

NON-SURGICAL ET CATHETER
(Martínez et al., 2002, 2004)

- Firm enough to pass the cervix folds
- Flexible enough to progress along the uterine horns
- 1.5 m length
- External diameter: 4 mm; Inner diameter: 0.7 mm


Tip of the transfer catheter
Tip of the insemination catheter
Cervical canal
Uterine body
Uterine horns
A
B
C
D
E
F




A
B
C
D
E


Laparoscopy


Laparotomy

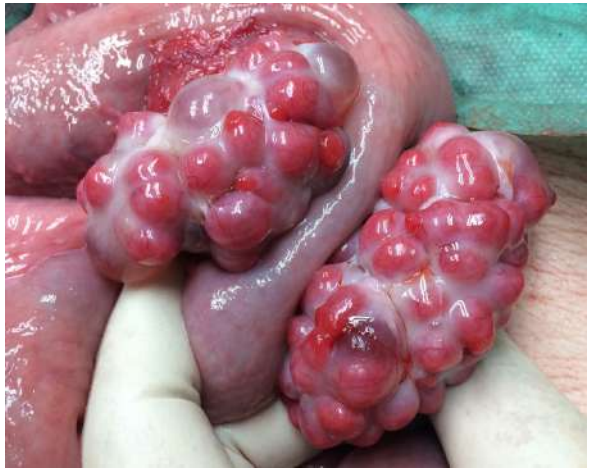
Sedation of the sows	No
Place	55.2±3.1 cm UH
Volume of medium	0.7 ml

	FRESH BLASTOCYSTS	FRESH MORULAE
	Ns-30	Ns-30
No. of recipients	111	25
Pregnancy rate (25 d), N (%)	83 (74.8)	23 (92)
Pregnancy rate (35 d), N (%)	79 (71.8)	23(92)
Farrowing rate, N (%)	79 (71.8)	23 (92)
Total born (mean±SD)	9.6±3.3	9.4±0.8
Born alive (mean±SD)	9.0±3.0	9.2±3.0
Piglet birth weight (mean±SD)	1.6±0.4	1.5±0.1
Piglet production efficiency (%)	22.8	28.8

Martinez et al., 2016. Reprod Dom Anim 51(1):123-9

Martinez et al., 2014. PLoS One 13;9(8):e104696.

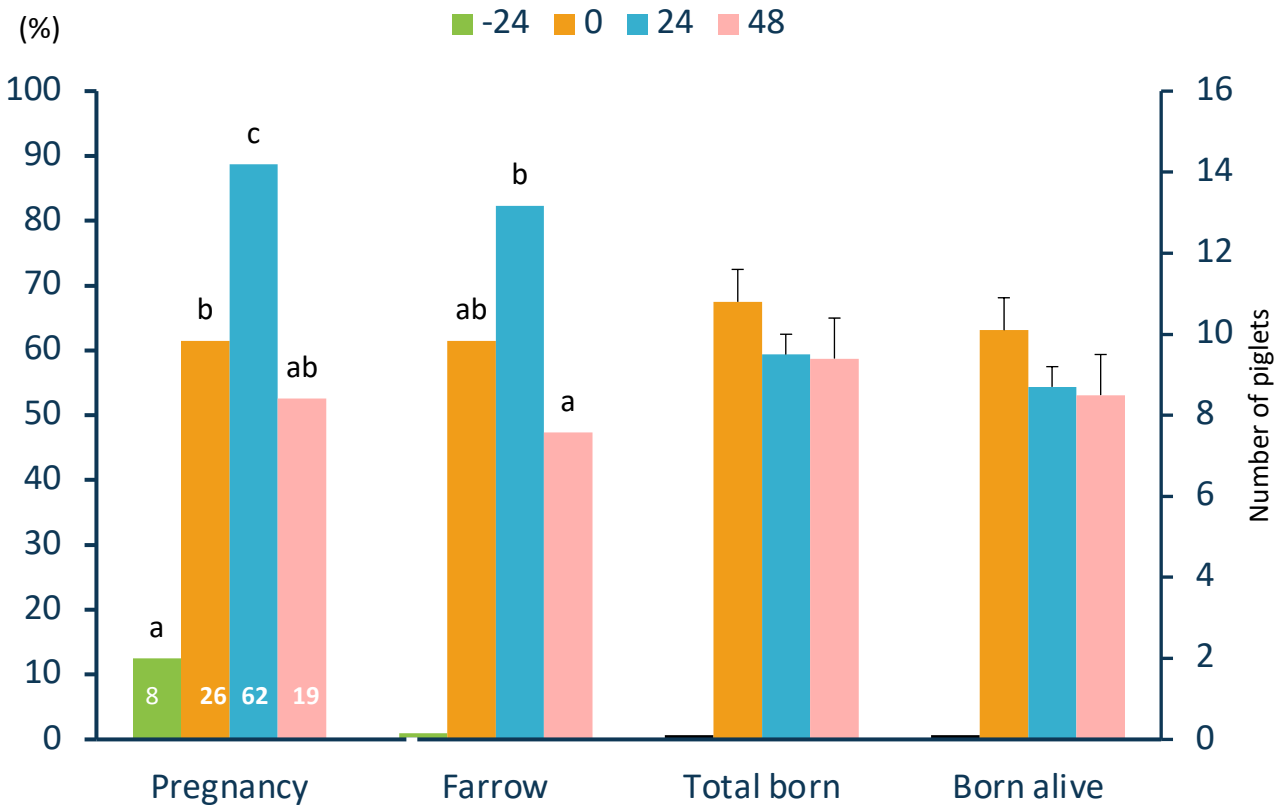
Some factors affecting the NS-ET results



- **Synchrony between donors and recipients**

Synchrony between embryo donor and recipients

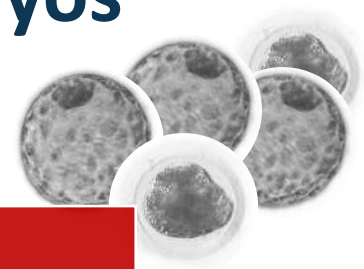
Recipients in estrus before (-) or after (+) donor sows



Angel et al., 2014. J Reprod Dev 60(5):371-6

[a,b,c,] within the same parameter P < 0.05

The best way to exchange genetic material ... the embryos



Limitations

EMBRYO TRANSFER

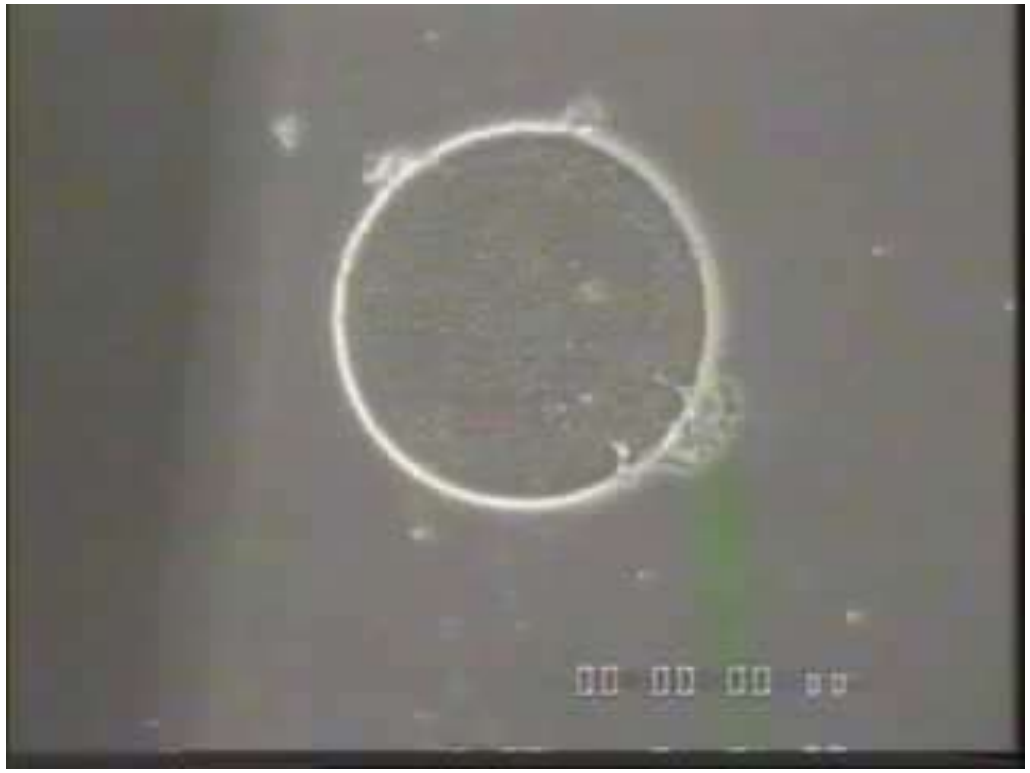
- Surgical embryo collection
- Non-surgical embryo transfer
- Development of vitrification

Embryo Vitrification

The main obstacles…

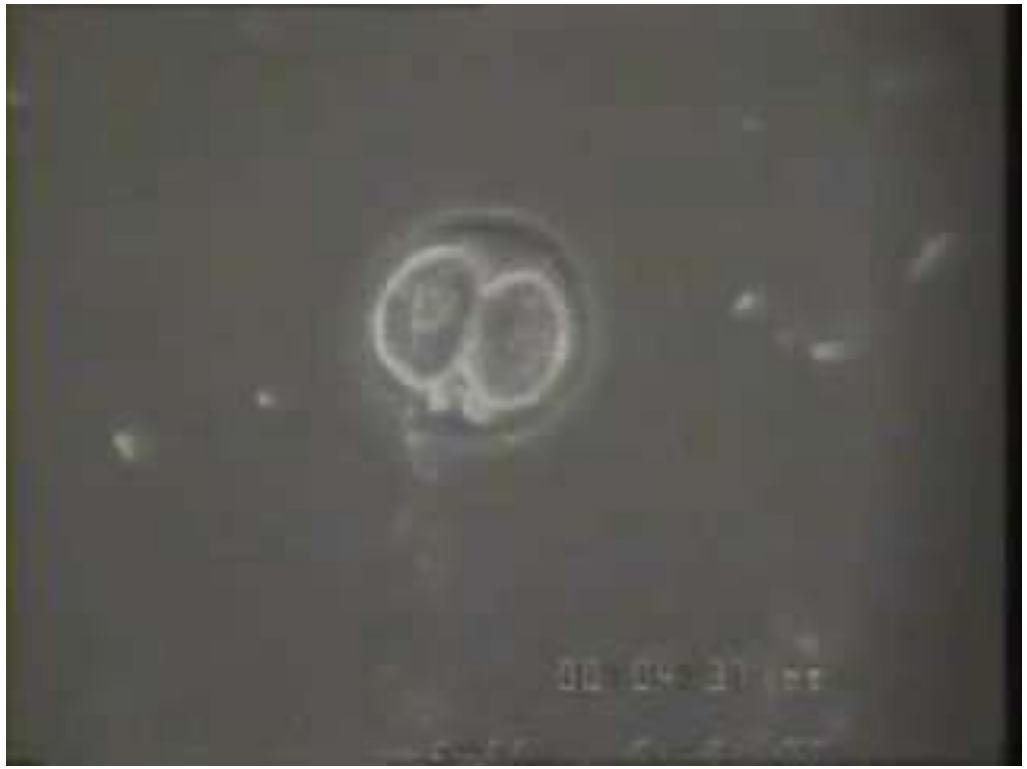
- High chilling injuries
- Surgical procedures for embryo collection and embryo transfer
- Expensive and time-consuming experiments

Vitrification (Rall and Fahy, 1985): transformation of water directly from the liquid phase into an amorphous phase or glass, while avoiding the formation of ice crystals



Traditional freezing

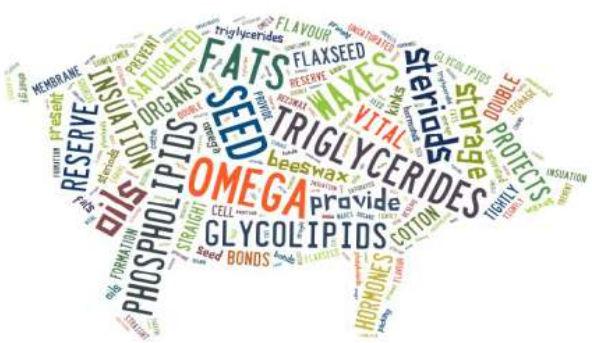
VS



Vitrification

Twenty years of porcine embryo cryopreservation...

HIGH SENSITIVITY OF PORCINE EMBRYOS TO CHILLING INJURY



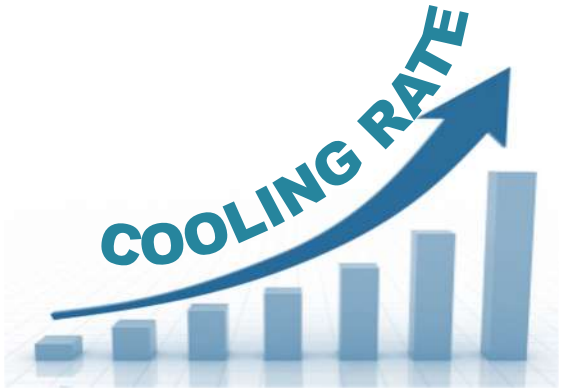
Traditional freezing

Vitrification

0.25 straws

Ultra-rapid vitrification

...



One of the lines of research in our laboratory seeks to improve the efficiency of porcine embryo vitrification

Some specific objectives:

- **Objective 1:** To evaluate different factor affecting the vitrification efficiency (media, cryoprotectant concentration, embryonic developmental stage, embryo donor…) in order to design an efficient vitrification protocol for porcine embryos.
- **Objective 2:** Development of a direct warming procedure to facilitate embryo transfer.
- **Objective 3:** Investigate the impact of vitrification on the embryo quality.
- **Objective 4:** To obtain pregnancies after non-surgical embryo transfers of vitrified embryos.
- **Objective 5**: To find a system for the simultaneous vitrification of a large number of embryos.

- **Effective vitrification protocol for porcine morulae and blastocysts without pretreatments.**

  Cuello et al., 2021. Front Vet Sci. 12;8:771996; Gonzalez-Plaza et al., 2022. Front Vet Sci 9:936753; Gonzalez-Plaza et al., 2023. Theriogenology 25;206:1-10

- **Reduced toxicity of vitrification solutions.**

  Cuello et al., 2008. Cryobiology 56(3):189-94; Sanchez-Osorio et al., 2008. Anim Reprod Sci 108(3-4):334-44.

- **Chemically defined media for embryo collection, vitrification, warming and transfer.**

  Cuello et al., 2016. Sci Rep 6:33915; Sanchez-Osorio et al., 2010. Theriogenology 73(3):300-8

- **Direct warming procedure.**

  Cuello et al., 2004. Theriogenology 62(6):1144-52;

- **Obtention of piglets after non-surgical transfer of vitrified morula and blastocysts**

  Cuello et al., 2005. Anim Reprod Sci 85:275-86; Gomis et al., 2012. Theriogenology 78(6):1339-49; Martinez et al., 2014. PlosOne 13;9(8):e104696; Martinez et al., 2015. Sci Rep 5:10587.

VITRIFICATION

1. WASHING TL-HEPES-PVA

1. WASHING TL-HEPES-PVA

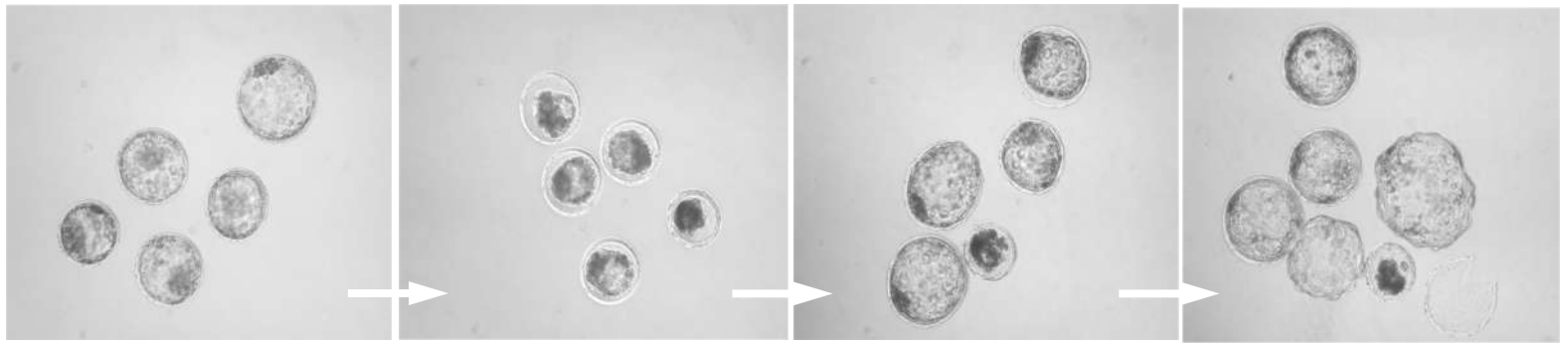
3. EQUILIBRATION V1: TL-HEPES-PVA + 7.5% DMSO + 7.5% EG (3 min)

4. EQUILIBRATION V2:
TL-HEPES-PVA + 16 % DMSO + 16 % EG + 0.4 M Sucrose (1 min)

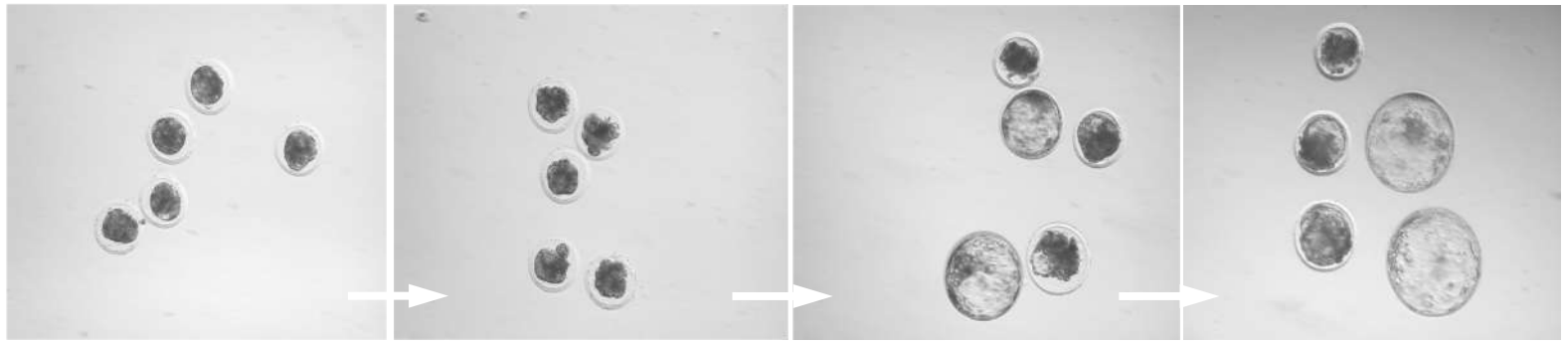
WARMING

TL-HEPES-PVA + 0.13 M sucrose
(5 min)

Superfine Open Pulled Straws (SOPS)



Fresh blastocysts | 0 h Post-warming | 24 h | 48 h



Fresh morulae | 0 h Post-warming | 24 h | 48 h

Survival Rate

>90%

80%

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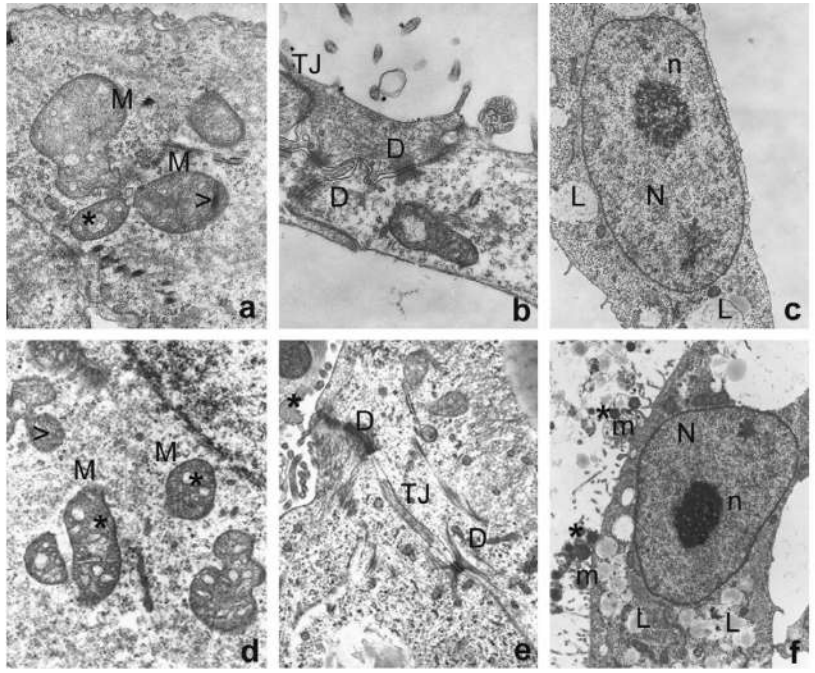
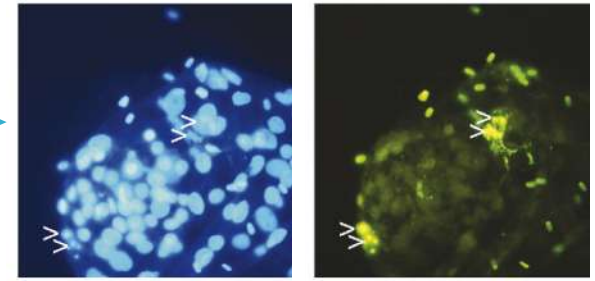


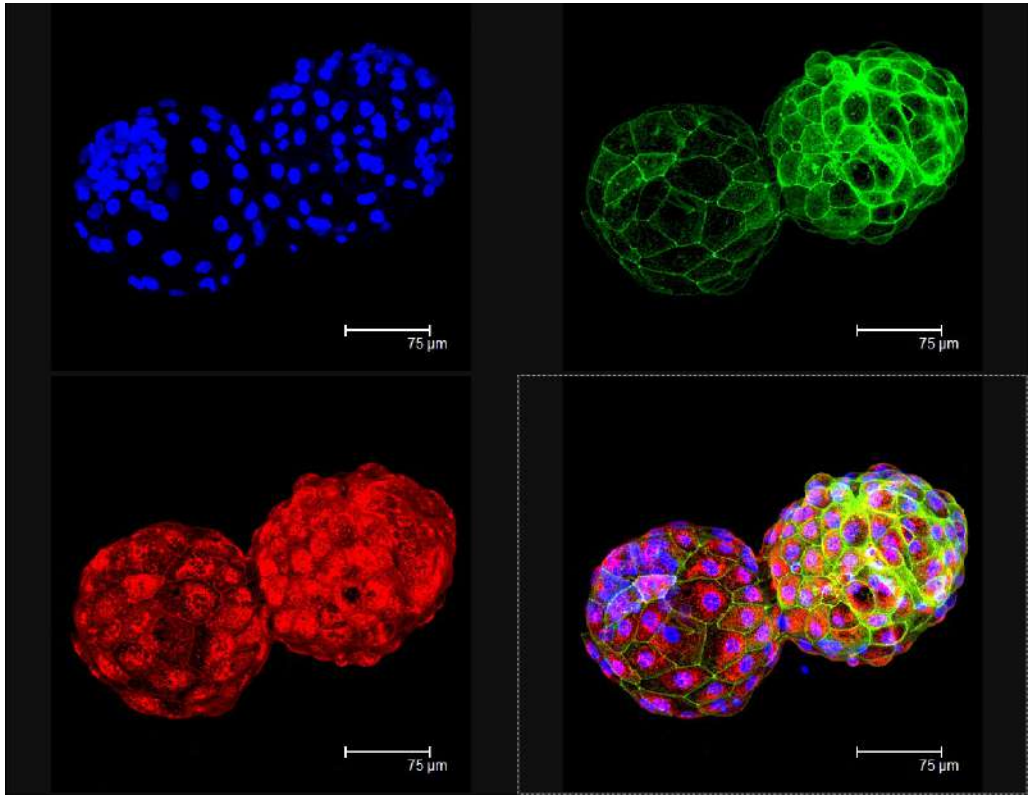
Cuello et al., 2006. Theriogenology 67:970-82.

Ultra-Structure
Number of cells
Apoptosis

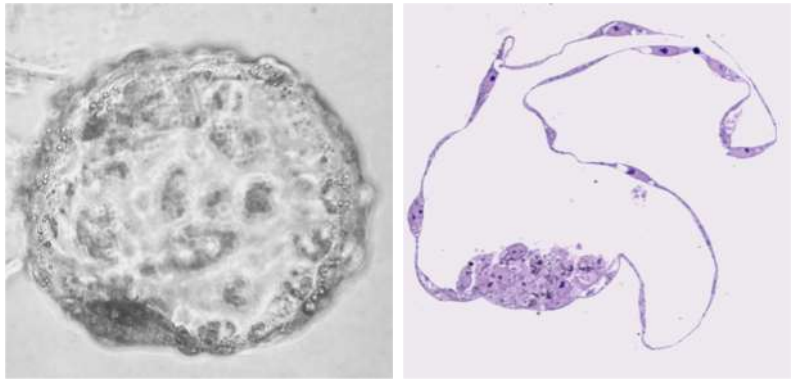


Cuello et al., 2006. Theriogenology 67:970-82.

Cytoskeleton structure

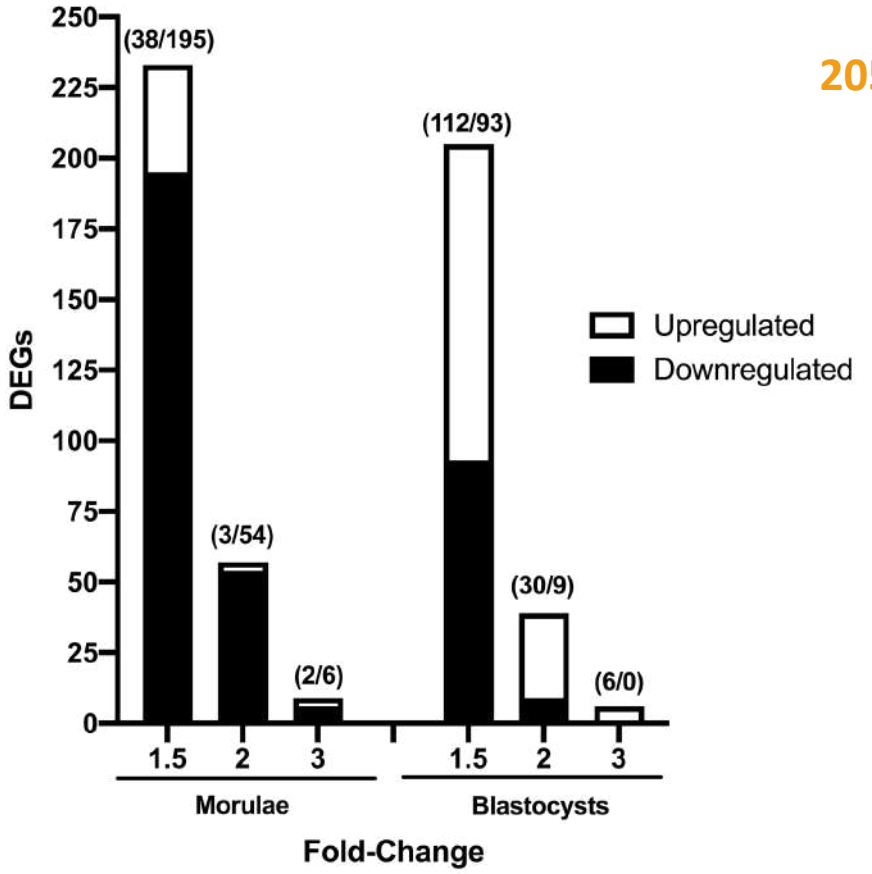


Cuello et al., 2010. Reprod Fertil and Dev 22(5) 808-817.



233 Differentially expressed genes in SOPS-vitrified morulae

205 Differentially expressed genes in SOPS-vitrified blastocysts



Moderate impact of vitrification in terms of number of differentially expressed genes and Fold-Change values.

Cuello et al., 2021. Int. J.Mol.Sci, 22,1222.

Cuello et al., 2021. Front. Vet. Sci.2021.771996

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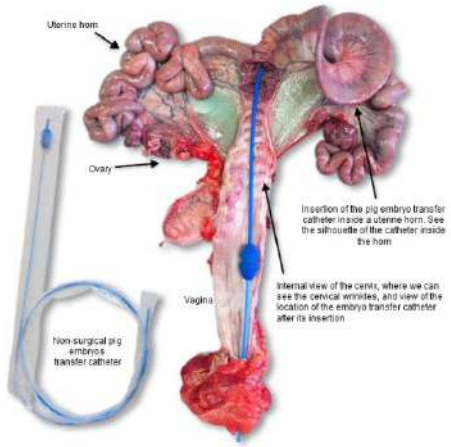
Reproductive parameters of the recipients after surgical and non-surgical deep intrauterine embryo transfer of vitrified-warmed porcine embryos.



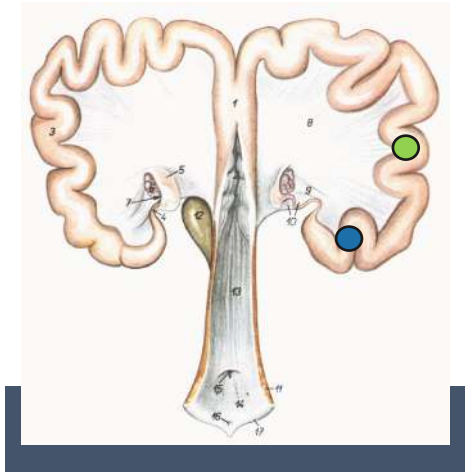
NsDU Transfer

OPS Vitrification

	Nº ET	Farrowing rate(%)	Litter size	Litter Efficiency (%)
Cuello et al., 2005	**21**	**42.98**	**5.4**	**2.’5**

Cuello et al., 2005. Anim Reprod Sci 85:275-86

Reproductive parameters of the recipients after surgical and non-surgical deep intrauterine embryo transfer of vitrified-warmed porcine embryos.



	Embryo transfer procedure*		
	S-30	NsDU-30	NsDU-40
No. of recipients	40	36	33
No. of parity	2.4±1.7	2.6±1.8	2.4±1.5
Pregnancy rate (25 d), N (%)	35 (87.5)[a]	17 (47.2)[b]	27 (81.8)[a]
Pregnancy rate (35 d), N (%)	30 (75.0)[a]	14 (38.9)[b]	25 (77.8)[a]
Pregnancy length (days) (mean±SD)	115.0±1.1	115.2±2.1	115.4±1.5
Farrowing rate, N (%)	30 (75.0)[a]	14 (38.9)[b]	24 (72.7)[a]
Total born (mean±SD)	9.6±2.7[a]	5.7±2.4[b]	9.9±2.1[a]
Born alive (mean±SD)	9.2±2.5[a]	5.5±2.4[b]	9.5±2.2[a]
Piglet birth weight (mean±SD)	1.5±0.3	1.6±0.4	1.4±0.2
Piglet production efficiency (%)	23.0[a]	7.1[b]	17.3[c]

* S-30: Surgical transfers with 30 vitrified-warmed embryos; NsDU-30: Non-surgical transfers with 30 vitrified-warmed embryos; NsDU-40: Non-surgical transfers with 40 vitrified-warmed embryos. [a,b,c] Different letters in the same row indicate differences (P<0.004)

Gomis et al., 2012. Theriogenology 78(6):1339-49;
Martinez et al., 2015. Sci Rep 5:10587.

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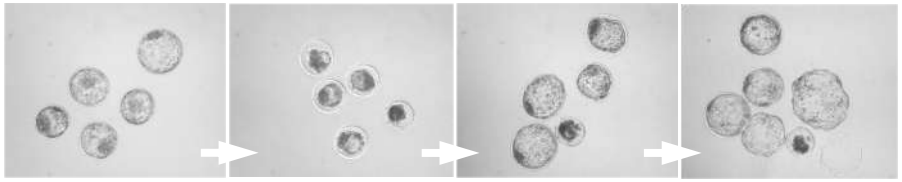
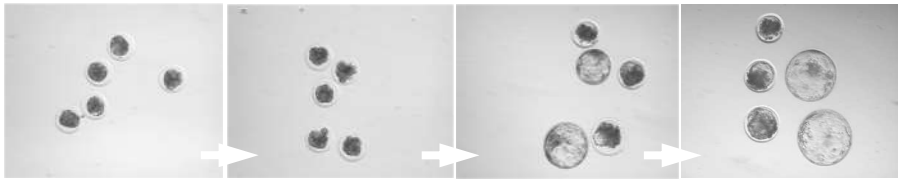




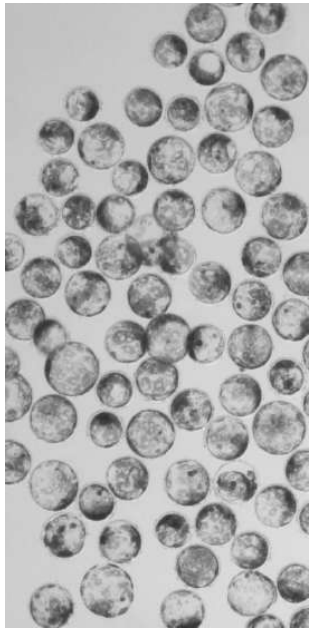
IN VIVO PRODUCED EMBRYOS

	SOPS (5-7 emb)		CRYOTOP (20 emb)
Survival Rate *in vitro*	>90%		>90%
	80%		>90%

Cuello et al., 2021. Front Vet Sci. 12;8:771996; Gonzalez-Plaza et al., 2022. Front Vet Sci 9:936753;
Gonzalez-Plaza et al., 2023. Theriogenology 25;206:1-10

IN VITRO PRODUCED EMBRYOS

	SOPS (5-7 emb)
Survival Rate	44.7% (forskolin)
in vitro	51.1% (ascorbic acid)
	~35% Control



Cuello et al., 2013. Anim Reprod Sci. 30;136(4):296-302; Nohalez et al., 2018. Theriogenology 113:113-119.

CONCLUSIONS

- Porcine in vitro embryo production is necessary for biomedical and reproductive research but still not appropriate for agricultural purposes.
- Vitrification allows the efficient long-term storage of porcine morulae and blastocysts with moderate impact on the embryo quality post-warming.
- Non-surgical embryo transfer together with vitrification allow the simple, cost-effective and safe exchange of genetic.
- Cryotop-vitrification allow the simultaneous vitrification of at least 20 blastocysts or morulae with excellent embryo survival post-warming.

IN SITU CONSERVATION MEETING
Toledo, May 22nd 2023

ANIMAL REPRODUCTION RESEARCH GROUP

Emilio A Martinez
Juan M Vazquez
Jose L Vazquez
María A Gil
Inmaculada Parrilla
María D Ortega
Alejandro Gonzalez-Plaza
Manuela García-Cánovas

UNIVERSIDAD DE MURCIA

DPTO. MEDICINA Y CIRUGÍA ANIMAL

FACULTAD DE VETERINARIA

f SéNeCa (+)
Agencia de Ciencia y Tecnología Región de Murcia

GOBIERNO DE ESPAÑA
MINISTERIO DE CIENCIA E INNOVACIÓN

IMIB
Instituto Murciano de Investigación Biosanitaria

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Twitter: Juliacolorea