UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Advances in *in Vitro* and *in Vivo* Porcine Embryo Production

Avances en la Producción *in Vitro* e *in Vivo* de Embriones Porcinos

Dª Alicia Nohalez Ruiz

2018

UNIVERSIDAD DE
MURCIA

Emilio Arsenio Martinez García, Catedrático del Departamento de Medicina y Cirugía Animal de la Universidad de Murcia y Cristina Cuello Medina, Profesora titular del Departamento de Medicina y Cirugía Animal de la Universidad de Murcia,

AUTORIZAN

A la Licenciada en Veterinaria Dña. Alicia Nohalez Ruiz a presentar la Tesis Doctoral titulada "Advances in in vitro and in vivo porcine embryo production" ("Avances en la producción in vitro e in vivo de embriones porcinos") ante la Comisión de Doctorado como compendio de publicaciones. Dicha Tesis ha sido realizada bajo nuestra dirección y reúne las condiciones legales precisas para optar al título de Doctor en Ciencias Veterinarias. La Tesis es un compendio de seis artículos publicados en revistas internacionales incluidas en el primer y segundo cuartil del JCR, todas ellas de gran difusión en el área de la Reproducción Animal. Los seis artículos presentan un cuerpo y unos objetivos comunes, aumentar la eficiencia para obtener blastocistos de buena calidad con el objetivo final de que sean transferidos a las receptoras, lo cual justifica la presentación en el formato de compendio de publicaciones. Este formato permite plasmar con facilidad el recorrido realizado por el doctorando y se ajusta al modelo de tesis presentadas actualmente dentro de nuestra área en el ámbito internacional.

Para que conste a los efectos oportunos, emitimos este informe en Murcia a 11 de Abril de 2018.

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TESIS DOCTORAL

POR COMPENDIO DE PUBLICACIONES

PhD THESIS BY PUBLICATIONS

Article 1

Effects of two combinations of cryoprotectants on the in vitro developmental capacity of vitrified immature porcine oocytes. **Nohalez A**, Martinez CA, Gil MA, Almiñana C, Roca J, Martinez EA, Cuello C. Theriogenology 2015; 84:545-52.

Article 2

Effects of meiotic inhibitors and gonadotrophins on porcine oocytes in vitro maturation, fertilization and development. Gil MA, **Nohalez A**, Martinez CA, Ake-Villanueva JR, Centurion-Castro F, Maside C, Cuello C, Roca J, Parrilla I, Martinez EA. Reprod Domest Anim 2017; 52:873-880.

Article 3

Exgenous ascorbic acid enhances vitrification survival of porcine in vitro-developed blastocysts but fails to improve the in vitro embryo production outcomes. **Nohalez A,** Martineza CA, Parrilla I, Roca J, Gil MA, Rodriguez-Martinez H, Martinez EA, Cuello C. Theriogenology 2018; 6:113-119.

Article 4

Factors of importance when selecting sows as embryo donors. **Nohalez A**, Martinez CA, Reixach J, Diaz M, Vila J, Colina I, Parrilla I, Vazquez JL, Roca J, Gil MA, Rodriguez-Martinez H, Martinez EA, Cuello C. Animal. 2017; 11:1330-1335.

Article 5

Eventual re-vitrification or storage in liquid nitrogen vapor does not jeopardize the practical handling and transport of vitrified pig embryos. **Nohalez A**, Martinez CA, Parrilla I, Roca J, Gil MA, Rodriguez-Martinez H, Martinez EA, Cuello C. 8;113:229-236

Article 6

The Recipients' parity does not influence their reproductive performance following non-surgical deep uterine porcine embryo transfer. Martinez EA, **Nohalez A**, Martinez CA, Parrilla I, Vila J, Colina I, Diaz M, Reixach J, Vazquez JL, Roca J, Cuello C, Gil MA. Reprod Domest Anim. 2016; 51:123-9.

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To whom it may concern

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DATE
March 20, 2018

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A comprehensive evaluation of the PhD Thesis from Alicia Nohalez Ruiz entitled:

Advances in *in vitro* and *in vivo* porcine embryo production

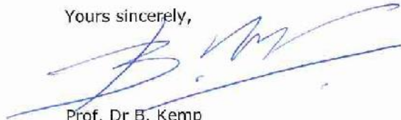
The aims of this thesis were to evaluate effects of different cryo-protectants, meiotic inhibitors, exogenous antioxidants, and re-vitrification on developmental capacity of oocytes and IVM, IVP, IVC outcomes. Moreover, donor characteristics in relation to reproductive and embryonic parameters and recipients characteristics in relation to NsDU-ET outcomes where assessed. These studies contribute to a higher success rate of IVP and allow better practical application of IVP in the pig. Improvement of IVP is urgently needed in the pig industry for breeding and sanitary reasons and also when the pig is used as an animal model for medical studies.

The obtained results are of great value. The presence of the meiotic inhibitor dbcAMP during the first halve of the maturation process markedly increases the capacity of oocytes to develop to the blastocyst stage. Addition of antioxidants to the vitrification and warming medium increases survival of in vitro produced blastocysts. Re-vitrification can be successfully applied in practice. Moreover, parity and weaning to oestrus intervals of donors and parity in recipients do not effect IVP results. These contributions are of great scientific interest but also for application in practice. The six papers are all published or accepted in reputable top journals for this field of science.

The thesis has a logical structure with a coherent concise literature review introducing the reader into the main aims of the thesis. The methods used in the experiments are relevant and up to date. The experiments are well executed and critically interpreted and discussed. The text is written very clearly and with the proper use of English.

This thesis is very good contribution to pig reproduction biotechnology both in terms of science and as a contribution to the field. Based om my evaluation I fully recommend this thesis for a doctoral degree and suitable examination.

Yours sincerely,



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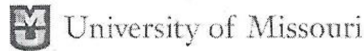

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March 21, 2018

To Whom It May Concern

I have reviewed the work performed by Ms. Alicia Nohalez Ruiz for her PhD thesis defense entitled "Advances in *in vitro* and *in vivo* porcine embryo production". The thesis is built upon six articles published in internationally ranked journals with a peer-review system. The thesis contains an introduction, set of general objectives, an extended summary including methodological considerations, experimental design and results followed by general conclusions, abbreviations and references.

In her thesis, the candidate has studied a topic which is current and up to date in the field of veterinary reproduction, particularly in reproductive biotechnology and of interest for an important species: porcine. This species is not only a vital agricultural animal, but also an essential biomedical research model, one that is physically and genetically close to humans. Compared to *in vivo* produced embryos, the efficiency to produce high quality embryos by *in vitro* maturation and fertilization is low. Besides, the embryo cryopreservation and embryo transfer are very challenging in this species as well because of its uniqueness in the high content of fatty acids in oocytes, its long uterine horns and it is litter-bearing. The thesis addressed a few critical and practical questions involved in oocyte maturation *in vitro*, oocyte freezing by vitrification and thawing/warming, and selection of embryo recipients. The amount of work is tremendous. The candidate has published six peer-reviewed research papers, with four as 1st author. The design of all experiments was always clear, reasonable and answered the hypothesis. Data were correctly analyzed and were clearly presented and well-illustrated. The meanings and the implications of the results were clearly discussed based on current literature.

Finally, current research work presented by the candidate would probably lead to new areas of research, which the candidate appears to be well-prepared for.

On the basis of the considerations above, I fully support Ms. Alicia Nohalez Ruiz's candidacy to the European Doctorate degree.

Dr. Jiude Mao

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April 10th, 2018

To whom it concern,

I have reviewed the Ph.D. thesis entitled "Advances in in-vitro and in-vivo porcine embryo production" prepared by Alicia Nohalez Ruiz. This thesis is a type built upon six articles (Alicia Nohalez Ruiz was the first author in four and the second author in two papers) published in *international peer-review journals*.

In this thesis, the candidate has conducted research to enhance the efficiency of the IVP of porcine embryos and to improve the porcine embryo transfer technology. In the first research, the candidate studied the effectiveness of two combinations of cryoprotectants on the in vitro developmental competence of vitrified immature porcine oocytes at the GV stage. In the 2nd research, the candidate evaluated effect of meiotic inhibitors and gonadotropins on in vitro maturation, fertilization and in vitro development of porcine oocytes and found that regardless of the presence of gonadotropins, the presence of dibutyryl cAMP during the first period of maturation may increase the capacity for developmental competence of the oocytes. In the 3rd research, the candidate found the addition of ascorbic acid to chemically defined Vitrification and warming media increased the survival rate of in-vitro produced porcine blastocysts. In the 4th research, the candidate demonstrated that fertilization rates and numbers of viable and transferable embryos collected at Day-6 of the cycle from super-ovulated donor sows were not affected by their parity, regardless of the time of the year. In the 5th research, more than 60% of vitrified embryos could be successfully re-vitrified and re-warmed. In the final research, the candidate showed that weaned sows from parity 1 to 5 are appropriate to be useful as recipients in non-surgical deep uterine embryo transfer. These experiments were well designed. Data was correctly analyzed, clearly presented and well interpreted. Results of these research were very valuable and contributed to improve the efficiency in in-vitro and in-vivo porcine embryo production.

On the basis of the considerations above, I fully recommend and support Alicia Nohalez Ruiz's candidacy to the European Doctorate.

Hiroaki FUNAHASHI, Ph.D.
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April 5th, 2018

To Whom It May Concern:
I am writing to provide my evaluation of Alicia Nohalez Ruiz's doctoral thesis titled "Advances in *in vitro* and *in vivo* porcine embryo production".

Swine embryo technologies have progressed significantly over recent years offering important applications for both porcine industry and research purposes. The implementation of the porcine embryo preservation and transfer protocols may allow the exchange of valuable genetic material with minimal risk of disease transmission and reduced costs. In addition, pig has also become the animal of choice for biomedicine thanks to its physiological characteristics which are similar to that of humans. For these reasons, a more efficient *in vivo* and *in vitro* embryo production (IVP) and an optimization of the procedures for embryo preservation and transfer in this species are greatly needed. Despite the advances made, still remain unresolved challenges in porcine IVP including insufficient cytoplasmatic maturation of IVM oocyte, high incidence of polyspermy, low quality of blastocysts resulting from IVM-IVF as well as embryo transfer and embryo preservation. The studies presented in this thesis have examined the effect of addition of the antioxidant ascorbic acid during IVP and vitrification and evaluated the effect of three reversible meiotic inhibitors and their interactions with gonadotrophins on the meiotic maturation and developmental competence. Furthermore, the studies also addressed important factors affecting embryo transfer efficiency, like the selection of sows as embryo donors and the selection of the recipients. Finally, the last work of the thesis explores the possibility of re-vitrifying *in vivo* derived morulae and blastocysts and to assess the efficacy of transport of embryos in a special containers, the so-called dry shippers (DSs).

The thesis is interesting, well prepared and provides very useful information in the field of porcine reproductive biotechnologies. The experimental design is fine and appropriate methods have been used. The results of the thesis have been published in six original research papers in international journals indexed in the Journal Citation Reports.
For all these reasons I think that the thesis has been excellently performed and I fully recommend a doctoral degree being awarded to Dr. Alicia Nohalez Ruiz.

Sincerely,

Prof.ssa Giovanna Galeati
Department of Veterinary Medical Sciences

UNIVERSITY OF CALIFORNIA, DAVIS

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TO WHOM IT MAY CONCERN

The present is to certify that Alicia Nohalez, from the Department of Animal Medicine and Surgery, Unit of Reproduction, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain, spent a 3-month training and research period in my laboratory at the Department of Animal Science, University of California, Davis, California, between September 1st and November 30th of 2015.

Alicia Nohalez joined our research group in embryology and conducted a period of training and research in gene expression during early embryo development, cloning and transgenic technologies, including CRISPR/Cas9 gene editing approach. She has shown willingness to learn as well as to incorporate herself into a new team of graduate students and researchers. She has shown high ability to deal with her tasks, learning capacity and open attitude for research. Alicia Nohalez is to be considered an asset to any research team elsewhere and she is certainly very welcome to return to our institution in the future.

In Davis, December 1st, 2015

Pablo J. Ross, PhD
Associate Professor

Universidade de Lisboa
Faculdade de Medicina Veterinária

TO WHOM IT MAY CONCERN

I do declare that Dr. **Alicia Nohalez Ruiz,** as a Doctoral trainee under my supervision, performed research and learned molecular biology techniques in the Laboratory of Reproduction, at the Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal, from 12th September until 16th December 2016. While here, she got training on protein extraction and Western Blot analysis on porcine endometrium and embryos.

This was a unique opportunity for knowledge transfer, and for the establishment of a long lasting inter-institutional and international collaboration between this laboratory in University of Lisbon and the University of Murcia.

Lisbon, 3rd February 2017

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El contrato-beca que me ha permitido realizar la presente Tesis fue otorgado por El Ministerio de Economía y Competitividad (BES-2013-064069). Los experimentos contemplados en la presente Tesis han sido financiados por el Ministerio De Economía y competitividad. Dirección general de Investigación Científica y Técnica (RTC-2016-5448-2, AGL2012-386219, AGL2015-69735-R) y Fundación Seneca (GERM 04543/07, 19892/GERM/15).

A mis padres, mis hermanos, y mis sobrinas.

A Pablo.

AGRADECIMIENTOS

A los profesores Emilio Martínez García y Cristina Cuello Medina, directores de esta Tesis. Por acogerme en su grupo y dedicar gran parte de su tiempo a mi aprendizaje, por su paciencia y dedicación y por la confianza que han depositado en mí. Es un orgullo formar parte de este gran equipo.

A los profesores Jordi Roca, María Antonia Gil Corbalán, Inma Parrilla y Xiomara Lucas, por su apoyo y por los conocimientos que me han transmitido.

A mis compañeros, Carolina, Miquel y Moisés por su cariño y su confianza y por haberme prestado su ayuda cuando la he necesitado.

A mis compañeras y amigas, Carmen Rodenas, Isabel Barranco, Cristina Perez y Lorena Padilla, por los grandes momentos que hemos vivido, porque juntas formamos un gran equipo y porque sé que puedo contar con ellas para todo.

A mi gran compañera y amiga, Cristina Martinez Serrano, por haber participado en esta tesis tanto como yo. Gracias por tu ayuda y apoyo incondicional, porque juntas hemos pasado grandes momentos que espero no se acaben jamás.

A mis amigas de Villamalea, Belinda, Mónica, Estefania, Mila y Rocio. Por ser mi desconexión del estrés y del trabajo. Siempre habéis formado y formaréis parte de mi vida.

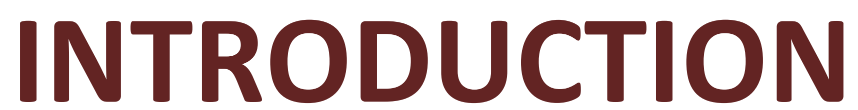
A Pablo, por ser la persona más importante de mi vida, porque juntos formamos un perfecto engranaje, un equipo imbatible capaz de vencer cualquier obstáculo. Gracias por ser como eres, por comprenderme y mimarme más que nadie y por confiar más en mí que yo misma.

A mis sobrinas, porque en tan poco tiempo se han convertido en parte esencial en mi vida y son las responsables de mi absoluta felicidad.

Y por último, a mi padre, a mi madre y a mis dos hermanos. A vosotros os debo todo lo que soy, habéis formado parte de cada uno de los momentos de mi vida, me habéis apoyado siempre, y me habéis dado fuerzas cada vez que lo he necesitado. Esta tesis es tan mía como vuestra. Gracias por ser la mejor familia que se puede tener. Solo veros felices es lo que me hace feliz. Os quiero.

INDEX

Introduction	1
Objectives	9
Extended Summary	13
Conclusions	33
Abreviations	37
Resumen general	41
References	71
Articles	81
Graphic appendix	91
Appendix	99


INTRODUCTION

INTRODUCTION

Swine embryo technologies have progressed significantly over recent years offering important applications for the porcine industry and also for research purposes. The implementation of the porcine embryo preservation and transfer protocols may allow the exchange of valuable genetic material with minimal risk of diseases transmission and reduced costs, which would be of great interest for the porcine sector. Pigs are not only crucial for meat production, but they are also significant for biomedicine thanks to its physiological characteristics, which are similar to that of humans (Rogers et al., 2008; Chorro et al., 2009; Vilahur et al., 2011). In this sense, porcine specie has emerged as the best biomedical model (Roura et al., 2016) and has been proposed as potential donor or producer of tissues and organs for humans. For these reasons, efforts towards more efficient *in vivo* and *in vitro* production (IVP) embryo systems and better procedures for embryo preservation and transfer in this species may have great impact in animal production and biomedical research areas.

The IVP of porcine embryos has presented numerous challenges to researches over the past decades (reviewed by Abeydeera et al., 2002, reviewed by Gil et al., 2010; reviewed by Grupen et al., 2014). The first piglets from IVP embryos were obtained by Mattioli et al., (1989). From then on, there have been numerous attempts to improve IVP techniques with progress in *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC; Reviewed by Gil et al., 2010). However, despite this extensive research, there are different impediments still unresolved. Current IVP systems still suffer from insufficient cytoplasmic abilities of *in vitro* matured oocytes, polyspermic fertilization and inappropriate culture conditions that lead to a poor quality of *in vitro* produced embryos and low efficiency of blastocyst formation.

From a practical point of view, a huge number of immature oocytes are required for IVP of porcine embryos. Immature oocytes are generally recovered from ovaries of slaughtered animals. However, not all the laboratories/research centers have a nearby available industrial slaughterhouse. An alternative to using oocytes from slaughtered gilts is the cryopreservation of pig oocytes, which could provide more flexibility to obtain available oocytes for IVP. Successful cryopreservation of porcine oocytes was first achieved by Isachenko et al., (1998), and since then, several researches have been focused on improving the oocyte cryopreservation protocols for this specie. In this aspect, vitrification decreases chilling injury

and it appears to be the best method to cryopreservation of porcine oocytes. Although matured oocytes are traditionally preferred for vitrification since survival is relatively high, their fertilization and developmental competence are seriously compromised (Egerszegi et al., 2013; Somfai et al., 2012). By contrast, cumulus cell–oocyte complexes (COCs) vitrified at the germinal vesicle (GV) stage show low survival rates but maintain their capacity to undergo normal fertilization (Egerszegi et al., 2013). The permeating cryoprotectans (CPAs) most commonly used for porcine oocyte cryopreservation are ethylene glycol (EG), glycerol (Taniguchi et al., 2011), dimethyl sulfoxide (DMSO; Gupta et al., 2007), propylene glycol (PG; Somfai et al., 2014), and acetamide (Fuku et al., 1995). Among them , EG, which has high permeability and low toxicity , has been shown to be the most effective CPA for porcine oocytes (Wu et al., 2006; Huang et al., 2008). Although the combination of EG and DMSO has been shown to be very effective in the vitrification of porcine embryos (Cuello et al., 2008), DMSO detrimentally affects the meiotic competence of GV stage porcine (Gupta et al., 2007) and murine (Trounson et al., 1989) oocytes. These investigations suggest that DMSO may not be adequate for oocyte vitrification; thus, PG could be an appropriate substitute for DMSO due to is more permeable in both porcine oocytes and embryos (Pedro et al., 2005). In this sense, we consider of interest to evaluate the effectiveness of the EG-DMSO and EG-PG CPA combinations for the vitrification of porcine immature oocytes.

Polyspermy , is one of the mayor concerns of the current porcine IVP protocols . The polyspermic fertilizations rate still remains at 30-50% in most laboratories (Funahashi and Nagai, 2000; Gil et al., 2004; Suzuki et al., 2000; Martinez et al., 2017). Multitude of studies (reviewed by Aveydeera ., 2002, reviewed by Funahashi ., 2003 and Kikuchi et al., 2002) have been carried out in efforts to identify the potential oocyte deficiencies or improve the IVF conditions . Exposure of the gametes , before or during IVF to oviduct fluid, cells (Kim et al., 1997; Nagai and Moor., 1990) or follicular fluid (Funahashi and Day., 1993) has been showed some benefit to improve the efficiency of fertilization. In addition, a time reduction of gametes co-culture (Grupen and Nottle., 2000; Funahashi and Romar., 2004; Gil et al., 2004) or modifications in the equipment used for IVP to regulate the number of penetrable spermatozoa close to the oocytes (Funahashi and Nagai., 2000; Wheeler et al., 2004) mimicking the physical conditions of IVF have also been studied . However , although these techniques have achieved moderate the frequency of polyspermy, it has not been eliminated completely yet. A critical point of the porcine IVP, which affects polyspermy and also the subsequent embryo development and quality is the insufficient cytoplasmic maturation of oocytes *in vitro*. It is known that oocytes from ovaries of slaughtered prepubertal gilts are at

different growth phases and developmental stages. Additionally, mammalian oocytes can restart meiosis spontaneously when they are removed from the follicle (Pincus and Enzmann., 1935), resulting in oocytes with asynchronous nuclear and cytoplasmic maturation. This nuclear-cytoplasmic asynchrony has been considered mainly responsible for the differences in developmental competence between *in vitro* matured porcine oocytes (Abeydeera., 2002). To resolve this issue, it is interesting the employment of synchronization techniques. These techniques are based on the use of reversible meiotic inhibitors (MINs) and have been reported for the oocytes from different species, including humans (Lange Consiglio et al., 2010; Leal et al., 2012; Nogueira et al., 2006). In swine, specific MINs such as the protein synthesis inhibitor cycloheximide (Le Beux et al., 2003; Ye et al., 2005; Ye et al., 2002), dibutyryl cyclic adenosine monophosphate (dbcAMP; Funahashi et al., 1997), the cellcycle-dependent kinase inhibitor roscovitine (Romar and Funahashi, 2006) and the phosphodiesterase inhibitors such as cilostamide (Dieci et al., 2013; Laforest et al., 2005; Sasseville et al., 2006) have obtained a completely reversible inhibition of meiosis. On the other hand, meiotic resumption is controlled by gonadotrophins (Gns). With all of this in mind, we decided to evaluate the interactions of MINs with Gns and their effects on IVP system.

Last but not least important concern is related with IVC conditions. Despite of the advances in IVC of porcine embryos, culture conditions are suboptimal and the efficiency is still low (reviewed by Gil et al., 2010; Somfai et al., 2011). The culture media most commonly used are the North Carolina State University (NCSU) media (NCSU-23 and NCSU-37), which were described by Petters and Wells, (1993). Although these media sustain the development of embryos to the blastocysts stage, embryos produced *in vitro* have lower developmental competence and quality than embryos cultured *in vivo* (reviewed by Kikuchi et al., 2004). To improve the IVP outcomes is essential to emulate *in vivo* conditions. One of the main differences between the *in vitro* and the *in vivo* conditions are related to the oxidative stress (Fisher and Bavister., 1993). The IVP environments have a higher oxygen content than those *in vivo*, which results in an increased of reactive oxygen species (ROS) production (Luvoni et al., 1996; Takashi et al., 2002). These high ROS levels during embryo IVP have been shown to be detrimental to gametes and embryos (Luberda., 2005; Silva et al., 2007; Kim et al., 2015). Consequently, protecting oocytes and embryos against oxidative stress during IVC is crucial for improving embryo IVP efficiency and embryo quality. To overcome this drawback, a commonly used strategy is the addition of antioxidants to the different IVP media (Tao et al., 2004; Funahashi., 2005; reviewed by Nagai et al., 2006; reviewed by Gajda., 2009; Kang et al., 2013). Within the different antioxidants, ascorbic acid (AsA) is one that provides mayor expectations.

AsA is the most important antioxidant in extracellular fluids (Warren et al., 2000) and it has been tested in embryo IVP, showing some beneficial properties. In porcine, AsA has shown positive effects on oocyte nuclear maturation (Tao et al., 2004), and blastocyst formation after parthenogenetic activation (Kere et al., 2013). Addition of AsA also seems to protect embryos against oxidative stress during IVC, improving embryo developmental competence after either IVF (Hossein et al., 2007) or parthenogenesis (Kere et al., 2013; Hu et al., 2012). In some studies, supplementation with AsA during IVC also enhanced the quality of blastocysts in terms of cell numbers (Hu et al., 2012) or survival after vitrification (Castillo-Martin et al., 2014). However, there is no systematic study exploring the influence of AsA on each step of the embryo IVP system and the presence of possible synergy effects.

The inefficiency of IVP holds up other reproductive techniques such *in vivo* derived embryo recovery. Although this technique is complex, costly and time-consuming can be an alternative to IVP when a high number of good quality embryos are required. A key factor to obtain numerous and good quality embryos is the selection of donors, which has received little attention in the field of porcine ET. Generally, in addition to genetic considerations, the selection of donors is usually based on good body condition, health status and breeding history. However, it is known that parity influences fertility and prolificacy, with higher farrowing rates and litter sizes in multiparous than in primiparous. Other essential factor in the reproductive performance of donors is the weaning estrus interval (WEI) due to it has an inverse relationship with length of estrus and time of ovulation (Soede and Kemp,, 1997; Belstra et al., 2004). Most weaned sows exhibit a fertile estrus between 3 and 5 days post-weaning. These sows with specific WEI are usually grouped and, for practical reasons, ET programs use these sows as donors. These differences in WEI could still influence the duration of estrus and the timing of ovulation. As a consequence, the number of non-transferable embryos at collection could increase due to the presence of uncompacted morulae (late ovulations) or hatched blastocysts (early ovulations), which are not advisable for ET because of their poor efficiency after transfers or by sanitary reasons, respectively. Other primary factor is the influence of season on the reproductive performance of sows. Compared with other times of the year, a longer WEI and lower fertility in summer is common in commercial swine production. Although a parity × season interaction has been also reported for estrus characteristics and ovulation in weaned sows (Knox and Rodriguez Zas., 2001), information is yet unavailable on how season affects embryo production in donor sows. These aspects of the donors (WEI, parity and season) were a subject of our study.

Regardless of whether embryos are obtained through either *in vivo* or *in vitro* techniques, the ultimate goal is embryo transfer (ET). Embryo transfer technology has been a demand of the pig industry for decades due to its numerous applications, particularly for the safe exchange of high-value genetic material with reduced transportation costs. Moreover, this technique considerably diminishes animal welfare problems. Despite these advantages, the commercial use of ET in pigs is still very limited when compared with other species (reviewed by Martinez et al., 2016). The main reasons for the limited use of ET in porcine is due to the requirement for surgical procedures and the difficulties in cryopreserving porcine embryos. However, in recent years, the situation has changed thanks to the improvement of vitrification procedures (Sanchez-Osorio et al., 2010) and the technological development of a safe non-surgical deep uterine (NsDU) ET (reviewed by Martinez., 2016).

In ET programs, the embryos must be stored from the time of their collection until they can be transferred to the recipients. Today, the only effective method for long-term preservation of porcine embryos is vitrification, which avoids the formation of ice crystals in the intracellular and extracellular spaces (Fuller et al., 2004). The improvement of vitrification procedures, specifically by using the super open pulled straw (SOPS) method (Vajta et al., 1997), has provided high *in vitro* post-warming embryo survival rates and hopefully higher pregnancy and farrowing rates after surgical ET of *in vivo*-derived porcine embryos vitrified without any previous treatment (reviewed Martinez et al., 2013). Although major progress has occurred in porcine embryo vitrification, many factors still remain unsolved. Of particular interest among these factors are embryo re-vitrification and air embryo shipment in the vapor phase of liquid nitrogen (LN_2). In some circumstances during ET, such as when the number of warmed embryos exceeds the number of embryos necessary to be transferred to the recipients or when some recipients cannot receive embryos due to health problems or difficulties during the insertion of the NsDU-ET catheter, a number of unexpected supernumerary embryos could have been warmed. Although these extra warmed embryos could be used to increase the number of transferred embryos per recipient, the ideal would be that they could be re-vitrified and stored again for future ET. In this sense, the possibility of re-vitrification should provide more flexibility for transfer to the respective recipients. However, the effects of re-vitrification on post-warming embryo survival rates in pigs have not yet been elucidated. On the other hand, it is known that the shipment of vitrified embryos in LN_2 for long distance transportation is forbidden by the International Air Transport Association (IATA). Therefore, the only option for the air transport of cryopreserved embryos is to use special containers, the so-called dry shippers (DSs). The DS Dewars have been used for cryopreserved

human semen (Lim et al., 2010; Punatanasakchai et al., 2008) and oocytes (Cobo et al., 2010) and for vitrified mouse embryos (AbdelHafez et al., 2011). However, to the best of our knowledge, there are no published studies on the effectiveness of DSs for the transport of vitrified porcine embryos.

Finally, it is important to mark the development of NsDU-ET, a simple, safe, effective, and practical procedure which can be performed under field conditions without specific facilities. Thanks to the improvements in NsDU-ET procedure, outstanding reproductive performance results have been achieved (Martinez et al., 2004; Angel et al., 2014a,b). However, despite the excellent results, there are many factors that can affect the reproductive performance of the recipients following NsDU-ET. Some of these factors have been extensively studied (Angel et al., 2014a,b; Martinez et al., 2014, 2015) such as the synchrony between the stage of embryo development and the recipients, the use of short-term and long-term stored embryos, and the number of embryos transferred per recipient. However, there are other factors that could affect NsDU-ET success, factors that must be assessment. One of these factors is the selection of recipients. In general, this selection is based on good body condition and health status. However, there are other factors of the recipients that can play a key role in the ET outcomes. For example, it is assumed that farrowing rates and litter sizes of primiparous sows are usually lower than those in multiparous sows and that the highest reproductive performance is apparently reached from parity 3 (Koketsu et al., 1999; Hughes and Varley., 2003), until parity 7 (Flowers and Alhusen., 1992; Hughes, 1998). Despite these observations, in all studies published to date on porcine ET, nulliparous (gilts), primiparous or multiparous sows were arbitrarily selected as recipients (Berthelot et al., 2001; Brüssow et al., 2000; Dobrinsky et al., 2000; Cameron et al., 2004; Martinez et al., 2014, 2015; Beebe et al., 2005, 2011; Cuello et al., 2005; Nakazawa et al., 2008) and none of these studies evaluated the effect of the recipients' parity on fertility and prolificacy post-ET. In addition, although the NsDU-ET catheter can be used in both gilts and sows (Martinez et al., 2004), the percentages of catheter insertion difficulties are lower in sows than in gilts. Moreover, the number of previous estrous cycles of the gilts is crucial to obtain adequate success of the insertions (Cuello et al., 2005). In addition, the use of recipient sows is often preferred because weaning is an excellent and natural procedure for estrus synchronization in swine (Britt et al., 1985). Therefore, we decided to investigate the effects of the recipient's parity on their reproductive performance after NsDU-ET.

OBJECTIVES

OBJECTIVES

The main objectives of this work were to enhance the efficiency of the IVP of porcine embryos and to improve the porcine ET technology. With this propose, the specific objectives contained in this thesis were:

1. To study the effectiveness of two combinations of CPAs, EG+DMSO or EG+PG, for the vitrification of GV stage porcine oocytes and their effects on the oocyte viability, fertilization ability, and the subsequent developmental competence (Article 1).

2. To evaluate the effect of three MINs (dbcAMP, cycloheximide and cilostamide) and their interactions with Gns on the meiotic maturation and developmental competence of porcine oocytes (Article 2).

3. To determine the effects of AsA supplementation to the IVM, IVF and IVC media, on the maturation, fertilization and embryonic developmental parameters, and to assess the effects of adding AsA to vitrification and warming defined media on the vitrification survival of IVP-porcine blastocysts (Article 3).

4. To investigate the effects of parity, season and WEI on the reproductive and embryonic parameters at day 6 after insemination of donor sows superovulated at postweaning estrus (Article 4).

5. To explore the possibility of re-vitrify *in vivo*-derived porcine embryos (morulae and unhatched blastocysts) (Article 5).

6. To assess if the DSs is adequate for maintaining the viability and quality of vitrified *in vivo*-derived porcine embryos (morulae and unhatched blastocysts) for a 3-day storage period (Article 5).

7. To investigate the effects of the recipients' parity on their reproductive performance after NsDU-ET (Article 6).

EXTENDED SUMMARY

EXTENDED SUMMARY

MATERIAL AND METHODS

All experimental procedures were performed in accordance with the 2010/63/EU EEC directive for animal experimentation and were reviewed and approved in advance by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain *(research code: 183/2015).*

Chemicals

All chemicals used in these experiments were purchased from Sigma-Aldrich Quimica SA (Madrid, Spain) unless otherwise noted.

Reagents and culture media

A physiological saline solution composed of NaCl 0.9% (w/v) and 70 µg/mL kanamycin was used to transport ovaries from the slaughterhouse to the laboratory. The cumulus-oocyte complexes (COCs) were collected and washed in Tyrode's lactate supplemented with 10-mM HEPES and 0.1% (w:v) polyvinyl alcohol (TL-HEPES-PVA; Funahashi et al., 2000; Martinez et al., 2014). The oocyte maturation medium was TCM-199 (Gibco Life Technologies S.A., Barcelona, Spain). The basic medium used for IVF was a modified Tris-buffered medium (mTBM: Abeydeera and Day, 1997) enriched with 2.0-mM caffeine and 0.2% (w:v) bovine serum albumin (BSA). A Dulbecco's PBS (DPBS; Gibco, Grand Island, NY) with 4 mg/mL of BSA was used for washing spermatozoa after thawing and before re-suspension in IVF medium. The embryo culture medium was NCSU 23 (Petters and Wells, 1993) supplemented with 0.4% BSA. The basic medium for vitrification and warming was the chemically defined TL-HEPES-PVA medium. The first vitrification medium (V1) was TL-HEPES-PVA containing 7.5% (v:v) of EG and 7.5% of DMSO and the second vitrification medium (V2) was TL-HEPES-PVA containing 16 % (v:v) of EG and DMSO, and 0.4 M sucrose. The warming medium consisted of TL-HEPES-PVA supplemented with 0.13 M sucrose. Embryo recovery media and ET media was TL-HEPES-PVA.

Culture conditions

Oocytes and embryos were cultured in an incubator at 38.5 °C with a 5% CO_2 in air and 95-97% of relative humidity unless otherwise noted.

COCs collection and IVM

Ovaries were collected from prepubertal gilts at a local slaughterhouse. The COCs were then collected using a sterile scalpel blade from the surfaces of medium-sized follicles (3-6 mm in diameter) in TL-HEPES-PVA. The COCs surrounded by two or more layers of compact cumulus cells were selected and washed in maturation medium. Groups of 75-80 COCs were transferred into each well of a four-well multidish containing maturation medium with hormones (eCG and hCG) for 22 hours. The oocytes were then incubated for another 20 to 22 hours in maturation medium without hormones.

***In vitro* fertilization**

After maturation, cumulus cells were removed with 0.1% hyaluronidase in maturation medium by vortexing for 2 minutes. The denuded oocytes were washed in IVM medium and in IVF medium. Then, groups of 30 oocytes were placed into 50 µL drops in IVF medium in Petri dish and were kept in the incubator until the spermatozoa were added. For IVF, cryopreserved and thawed semen from a mature boar was used. Just after thawing, 100 µL of sperm was washed in DPBS. The resulting pellet was suspended in IVF medium. After appropriate dilution, 50 µL of this sperm suspension was added to the medium with the oocytes such that final ratio was 1000:1 spermatozoa/oocyte. Finally, oocytes were co-incubated with spermatozoa for 5 hours.

***In vitro* culture**

After gamete co-incubation, presumptive zygotes were washed in IVC medium to remove spermatozoa that were not bound to the zona pellucida. Presumptive zygotes were then transferred (30 zygotes per well) into a four-well multidish containing glucose-free embryo culture medium that was supplemented with 0.3-mM pyruvate and 4.5-mM lactate for 2 days, and then changed to fresh embryo culture medium containing 5.5-mM glucose for an additional 5 days period.

Assessment of oocyte IVM, IVF parameters and embryo development parameters

To evaluate the maturation and fertilization parameters, some oocytes and presumptive zygotes were fixed at 44 of IVM and 18 hours after IVF, respectively, and stained with 1% (w:v) lacmoid. Oocytes with a chromatin enclosed in a nuclear membrane or oocytes with a condensed chromatin but without extruded polar body were classified as immature oocytes. Oocytes were considered mature when their chromosomes were organized at metaphase and they showed an extruded first polar body (MII). Presumptive zygotes were

considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei and two polar bodies. The penetration rate was considered as the ratio of the number of penetrated oocytes relative to the total number of mature oocytes inseminated. The monospermic rate was calculated as the ratio of oocytes with one female pronucleus, one male pronucleus and two polar bodies to the total number of matured oocytes penetrated. The efficiency of fertilization was the ratio of the number of monospermic oocytes relative to the total number of matured oocytes inseminated.

The cleavage (ratio of the number of embryos cleaved to two to four cells out of the total number of oocytes inseminated) and blastocyst formation rates (ratio of the number of blastocysts out of the total number of cleaved embryos) were morphologically evaluated at Day 2 and Day 7 post-insemination (Day 0), respectively. The total efficiency was described as the percentage of the total number of inseminates oocytes that reached the blastocyst stage. Blastocysts were fixed in 4% (v:v) paraformaldehyde in PBS to assess the total cell number. After fixation, embryos were washed with PBS and placed on a slide in drops of 4 µL of VECTASHIELD (Vector Labs, Burlingame, CA, USA) containing 10 µg/mL Hoechst 33342. The total number of nuclei that showed blue fluorescence was counted.

Vitrification and warming of oocytes and embryos

Vitrification was performed according to the method described previously (Cuello et al., 2008). Briefly, groups of 5 to 6 embryos/oocytes were washed in TL-HEPES-PVA and equilibrated in V1 during 3 minutes and V2 during 1 minute for vitrification. During the final equilibration, embryos/oocytes were placed in a 1 µL drop and loaded in the narrow end of a SOPS (Minitüb, Tiefenbach, Germany) by capillary action. Subsequently, straws were plunged into LN_2. After storage in LN_2, the straws were removed and warmed by the one-step warming method (Cuello et al., 2004). Briefly, the straws were vertically submerged in a well containing warming medium for 5 minutes. Then, vitrified-warmed oocytes or embryos were washed in TL-HEPES-PVA and cultured in maturation medium or in culture medium, respectively.

Oocyte viability assessment

Oocyte viability was evaluated using fluorescein diacetate (FDA) staining as described by Mohr and Trounson., (1980). The working solution was prepared by a 1:2000 dilution of the FDA stock solution in DPBS to reach a final concentration of 2.5 mg/mL of FDA. The COCs were stained in 500 mL of this solution for 2 minutes at 37 °C in a dark room, and finally, washed three times in DPBS. Oocytes were evaluated with ultraviolet irradiation using a GFP-II filter under an inverted fluorescence microscope. Live oocytes exhibited bright green fluorescence,

and FDA viability was calculated as the percentage of live oocytes of the total number of oocytes evaluated.

Evaluation of *in vitro* development post-warming

Morulae that had progressed to the blastocyst stage during the IVC and blastocysts that reformed their blastocoelic cavities post-warming with an excellent or good appearance were considered viable. *S*urvival rate was determined as the ratio of viable embryos at 24 hours of IVC to the total number of cultured embryos.

Differential staining of blastocysts

The number of inner cell mass (ICM) and trophectoderm (TE) cells of the vitrified-warmed blastocysts was determined using an indirect immunofluorescence protocol based on that described by Wydooghe et al., (2011). Briefly, blastocysts were fixed with 4% of paraformaldehyde and permeabilized. Then, blastocysts were incubated first in a 2 N HCL solution for 20 minutes, and after in 100 mM Tris-HCl (pH 8.5) for 10 minutes. After washing with PBS, blastocysts were incubated for 6 hours in blocking solution. Then, blastocysts were washed and incubated with the primary CDX-2 antibody (1:200; BioGenex, CA, USA) and with donkey anti-mouse IgG-Alexa Fluor® 568 conjugate (1:1000 in blocking solution; Invitrogen, Rockford, USA). Finally, blastocysts were placed on a slide in 4 µL of Vectashield (Vector Labs, Burlingame, CA, USA) containing 10 µg/mL Hoechst 33342 and total cell number (blue fluorescence) and TE cells (red fluorescence) were counted.

Measurement of intracellular GSH and ROS levels

Intracellular GSH and ROS levels of blastocysts were determined by staining with CellTraker Blue (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; CMF2HC; Invitrogen, ThermoFisher scientific, Massachusetts, USA) and H2DCFDA (2’, 7’-dichlorodihydrofluorescein diacetate; Invitrogen), respectively. Blastocysts were incubated in 10 µM of CellTracker Blue and 10 µM of H2DCFDA. Fluorescence was immediately observed with fluorescence microscopy with ultraviolet filters (370 nm for GSH and 460 nm for ROS). The fluorescence intensities of each blastocyst were analyzed by ImageJ software (Version 1.51h; National Institutes of Health, Bethesda, MD, USA).

Assessment of cellular apoptosis

Apoptotic cells were stained by terminal deoxynucleotidyl transferase and fluorescein dUDP nick-end labelling (TUNEL) as described in Brison et al., (1999) and Byrnee et al.,(1997)

using an APO-BrdUTM TUNEL Assay Kit (A23210; Invitrogen, Oregon, USA). Briefly, viable blastocysts were fixed in 4% paraformaldehyde and permeabilized. Positive control was incubated in DNase. Then, both control and experimental embryos were incubated in TUNEL reaction droplets. Finally, under a fluorescence microscope, nuclei stained with green fluorescence (TUNEL positive apoptotic cells) were counted. The percentage of apoptotic cells was calculated as TUNEL positive nuclei out of the total number of nuclei stained with Hoechst (apoptotic index).

Embryo donors and recipients

Sows (parity 2 to 6) with normal weight and good sanitary status were used in the experiments. Sows were individually allocated into crates in a mechanically ventilated confinement facility. Animals were fed a commercial ration according to their nutritional requirements. All sows had water available *ad libitum*. Boars were housed in individual pens in a commercial artificial insemination center (AIM Iberica, Murcia, Spain).

Detection of estrus, artificial insemination, embryo recovery and embryo assessment

The embryo donors were examined (beginning the day after weaning) for estrus twice a day by exposing sows to a mature boar and applying back pressure. Sows in estrus were inseminated with insemination doses previously prepared. Surgeries were performed by mid-ventral laparotomy on day 6 of the estrous cycle, considering day 0 to be the onset of estrus. Sows were previously sedated with azaperone (Stresnil®, Landegger Strasse, Austria; 2 mg/kg body weight, i.m.). General anesthesia was induced with sodium thiopental (B. Braun VetCare SA, Barcelona, Spain; 7 mg/kg body weight, i.v.) and maintained with isoflurane (IsoFlo®, Madrid, Spain). The reproductive tract was exposed and the corpora lutea in each ovary were counted. Embryos were collected as described previously (Martinez et al., 2014) by flushing the tip of each uterine horn with 30 mL of TL-HEPES-PVA. The recovered embryos were evaluated under a stereomicroscope for developmental stage and quality. One-cell eggs or poorly developed embryos were considered unfertilized oocytes or degenerated embryos, respectively. Unhatched embryos in morulae and blastocyst stage with an appropriate morphology according to the criteria determined by the International Embryo Transfer Society (IETS; Stringgfellow and Seidel., 1998) were classified as viable and were used in the experiments.

Non-surgical deep uterine Embryo Transfer

The NsDU-ETs were conducted in non-hormonally treated recipient sows using a previously described method (Angel et al., 2014a). Briefly, prior to transfer, each recipient

received a single intramuscular injection of a long-acting amoxicillin suspension (Clamoxyl LA; Pfizer, Madrid, Spain) at a dosage of 15 mg/kg. Recipients were housed in gestation crates in a small room exclusively used for that purpose. The perineal area of the recipients was thoroughly cleaned, and the vulva was then washed and decontaminated with chlorhexidine. NsDU ET catheters (Deep Blue ET catheter, Minitub, Tiefenbach, Germany) were used for the transfers. When the catheter was completely inserted into one uterine horn, a 1 mL syringe containing the embryos in 0.1 mL of TL-HEPES-PVA medium was connected to the catheter, and the contents were introduced into the catheter. An additional volume of 0.3 mL of TL-HEPES-PVA medium was used to force the embryos out of the catheter into the uterus.

Statistical analysis

Statistical analysis was performed using the IBM SPSS 19 statistics package (SPSS, Chicago, IL, USA), and differences were considered significant at $P < 0.05$. The results were expressed as the mean ± SD (standard deviation) or mean ± SEM (standard error of de mean). The percentage data were compared using Fisher's exact test. Binary variables (mean ± SD) and continuous variables were evaluated using the Kolmogorov–Smirnov test to assess the assumption of normality. Means of more than two groups were compared using a mixed-model analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Pairwise comparisons of means were performed using Student's t-test. The effects of season, parity, and WEI were evaluated using linear mixed effects models. Multivariable logistic regression analysis with the presence or absence of cysts on ovaries as the dependent variable was used to evaluate the odds ratios (ORs) and the corresponding 95% confidence intervals (CIs) for the independent variables of parity, season and WEI.

EXPERIMENTAL DESIGN AND RESULTS

OBJETIVE 1. To study the effectiveness of two combinations of CPAs, EG + DMSO or EG + PG, for the vitrification of GV stage porcine oocytes and their effects on the oocyte viability, fertilization ability, and the subsequent developmental competence (Article 1).

Experiment 1. Assessment of porcine oocytes viability after equilibration in EG+DMSO or EG+PG and the subsequent embryo develempoment.

Experimental design

We evaluated in 3 replicates the toxicity of the vitrification media prepared with two diffeent CPA combinations (EG + DMSO or EG + PG, groups). Oocytes were subjected to equilibration in the vitrification and warming solutions, but the oocytes were not vitrified. Oocyte viability (FDA viability) was examined by FDA staining two hours after treatment, and live oocytes were matured *in vitro*. Oocytes with lysed cytoplasmic membranes at 44 hours of maturation were considered to be degenerated and were excluded. At 18 hours after IVF, some presumptive zygotes were fixed to assess the fertilization parameters. The remaining presumptive zygotes were culture *in vitro*, and the developmental parameters of the embryos derived from oocytes exposed to CPAs were compared with control group.

Results

The FDA viability of the oocytes after 2 hours of equilibration in EG + DMSO and EG + PG-based media was very high (95.7 ± 0.4% and 95.6 ± 1.1%, respectively) but lower ($P < 0.01$) than that observed in the control group (99.5 ± 0.8%). Higher ($P < 0.05$) proportions of live oocytes from the EG + DMSO (18.1 ± 2.3%) or EG + PG (19.4 ± 2.6%) groups were degenerated at 44 hours of maturation compared with those in the control group (7.6 ± 1.3%). At 18 hours after IVF, the numbers of immature oocytes (< 12%) and degenerated oocytes (< 3%) were similar for all groups. No differences were observed in penetration (range 74.5 ± 2.4% -77.5 ± 2.6%), monospermy (range: 46.4 ± 4.1% - 57.5 ± 3.4%) and efficiency (range: 36.7 ± 3.0% - 49.2 ± 2.2%) between groups. The exposure of oocytes to the CPAs neither affected their developmental ability in terms of cleavage (range: 58.1 ± 3.3% - 61.6 ± 3.2%) and blastocyst formation rates (range: 29.3 ± 3.0% - 36.9 ± 2.9%) nor the total cell number (range: 44.6 ± 3.3 - 47.6 ± 3.3) in the blastocysts.

Experiment 2. Assessment of porcine oocytes viability after vitrification in EG+DMSO or EG+PG and the subsequent embryo development.

Experimental Design

The second experiment was designed to evaluate oocyte viability, fertilization parameters and embryo developmental competence of GV oocytes (n=1198) vitrified using either EG + DMSO or EG + PG.

A total of 4 replicates were performed. Oocytes were subjected to equilibration in vitrification media and vitrified and warmed as described above. Non-vitrified oocytes were used as controls. The treated and control oocytes were selected and evaluated as described in Experiment 1. Some of the oocytes were fixed 18 hours after IVF to assess their fertilization parameters. The rest of de presumptive zygotes were cultured. Cleavage rate, blastocyst formation and blastocyst total cell number.

Results

Vitrification reduced ($P < 0.001$) the FDA viability (67.0 ± 2.3% and 57.6 ± 2.3%; EG+DMSO and EG+PG, respectively) 2 hours after warming compared with control oocytes (97.3 ± 0.7%). The FDA viability was higher ($P < 0.01$) when the oocytes were vitrified using EG + DMSO instead of EG + PG. Similar proportions of live oocytes from the EG + DMSO and EG + PG groups were degenerated at 44 hours of maturation (59.8 ± 2.3% and 56.2 ± 2.6%, respectively); these proportions were higher ($P < 0.001$) than those observed in the control group (1.6 ± 1.3%). These degenerated oocytes were removed before IVF. At 18 hours after IVF, a higher proportion of oocytes from the EG + DMSO group (53.3 ± 5.0%) was considered degenerated compared with the EG + PG and control groups (9.2 ± 0.3% and 2.4 ± 0.9%, respectively). The percentage of immature oocytes was higher ($P < 0.05$) in the EG ± DMSO (25.0 ± 5.8%) group than the control (8.8 ± 1.7%) and EG + PG (17.7 ± 3.6%) groups. No differences in penetration (range: 57.0 ± 11.3% - 73.3 ± 2.8%) and monospermy (range: 63.3 ± 2.3% - 67.9 ± 5.0%) were observed between groups. However, oocytes vitrified using EG + DMSO exhibited lower ($P < 0.01$) efficiency rates (26.3 ± 7.7%) after IVF compared with the control and EG + PG group (42.0 ± 2.2% and 39.6 ± 2.4%, respectively. The total cell numbers per blastocyst (range: 32.8 ± 4.4 - 53.1 ± 2.6) was not affected by vitrification. However, the efficiency was higher in control group (31.9 ± 1.0%) than in EG + DMSO (6.6 ± 2.5%) and EG + PG (4.7 ± 1.6%) groups. In addition, cleavage rate and blastocyst formation were higher ($P < 0.001$) in the control group (53.4 ± 2.7% and 61.8 ± 5.3%, respectively) than in the vitrified

groups (25.9 ± 3.5% and 24.1 ± 10.0% in EG+DMSO group; 20.2 ± 5.8% and 27.2 ± 8.5% in EG + PG group).

OBJECTIVE 2. To evaluate the effect of three MINs (dbcAMP , cycloheximide and cilostamide) and their interactions with Gns on the meiotic maturation and developmental competence of porcine oocytes (Article 2).

Experiment 1. Interactive effects of MINs and Gns on nuclear maturation of oocytes.

Experimental design

This experiment was designed to evaluate the effects of the three MINs and their interaction with Gns on meiotic maturation. A total of 2058 oocytes were matured for 22 hours in the absence (control) or presence of dbcAMP (1 mM), cilostamide (20 µM) or cycloheximide (7 µM) with or without Gns, and for another 22 hours in the absence of MINs and Gns. Nuclear progression was assessed at 22 and 44 hours of maturation.

Results

The presence of MINs affected ($P < 0.005$) the proportion of oocytes in the GV and intermediate stages at 22 hours of maturation. In contrast, the supplementation of Gns and the MIN-Gn interaction had no significant effect on these parameters. In the absence of MINs, the Gns produced a decrease ($P < 0.02$) in the proportion of oocytes remaining at the GV stage. Yet, most oocytes cultured in the presence of MINs were arrested at the GV stage (range: 93.1 ± 5.7% - 99.4 ±1.2%) regardless of the presence of Gns in the medium. At 44 hours, the rates of oocytes reaching MII in the groups cultured with Gns were higher ($P < 0.03$) compared with those without Gns regardless of the presence or absence of MINs during culture. Only 46.6 ± 13.2% of the oocytes cultured in the absence of Gns and MINs achieved the MII stage by the end of culture, and the addition of MINs to the maturation medium was insufficient to increase that percentage. In the absence of Gns, the cultures supplemented with cilostamide showed a larger number of oocytes at the GV stage than the controls (80.3 ± 8.3% and 41.9 ± 11.5%, respectively). In the presence of Gns, the supplementation of the maturation medium with MINs failed to increase the rates of nuclear maturation.

Experiment 2. Interactive effects of MINs and Gns on developmental competence of porcine oocytes.

Experimental design

The second experiment was designed to evaluate interactive effects of MINs and Gns on developmental competence of oocytes. In this experiment, 2515 oocytes matured as in experiment 1 were inseminated and cultured to evaluate their developmental competence. In each replicate, a random subset of presumed zygotes was fixed and stained at 18 hours post-insemination (n=1155) to assess fertilization parameters. The remaining presumed zygotes (n=1360) were cultured to assess the *in vitro* embryo development at 2 and 7 days of culture.

Results

The presence of MINs, Gns and the interaction between both factors ($P < 0.01$) affected the penetration rate and the efficiency of fertilization of the inseminated oocytes. In the presence of Gns, only oocytes cultured in the presence of dbcAMP resulted in a higher ($P < 0.05$) penetration rate (95.5 ± 6.5%) and efficiency of fertilization (49.4 ± 6.4%) compared with those cultured with Gns in the absence of MINs (72.6 ± 7.1% and 33.7 ± 5.0%, respectively).

In the absence of Gns, although oocytes cultured with dbcAMP and cycloheximide showed higher ($P < 0.05$) penetration rates (80.9 ± 7.8% y 81.7 ± 14.4%) than oocytes cultured without MINs (56.5 ± 7.3%), no difference was observed in the efficiency of fertilization (23.4 ± 2.8%, 30.0 ± 3.7% and 19.6 ± 2.9%, respectively). Again, decreased penetration rates and efficiency of fertilization ($P < 0.05$) were observed in the group of oocytes cultured in the presence of cilostamide without Gns (27.2 ± 11.1% and 12.0 ± 6.4%, respectively) compared with oocytes cultured in absence of MINs and Gns (56.5 ± 7.3% and 19.6 ± 2.9%, respectively).

The presence of MINs, Gns and the interaction between both factors affected ($P < 0.05$) all of the embryo development parameters evaluated. The presence of dbcAMP during maturation increased ($P < 0.05$) blastocyst formation rates and the efficiency of blastocyst formation in both the presence (59.9 ± 7.4% and 45.1± 7.7%, respectively) and absence (40.8 ± 15.0% and 22.3 ± 8.6%, respectively) of Gns compared with the controls (38.9 ± 11.4 % and 26.3 ± 8.6% with presence of Gns y 18'8 ± 3'1% and 6'2 ± 2'3% with absence of Gns). Moreover, the oocytes cultured with dbcAMP and Gns had a higher ($P < 0.001$) efficiency of blastocyst formation (45.1 ± 7.7%) compared with the other treatment groups (15.6 ± 10.9% and 27.4 ± 6.8%, cycloheximide and cilostamide, respectively). No differences were observed in the total number of blastocyst cells among the groups (range: 31.7 ± 2.8% - 46.3 ± 5.8%).

OBJECTIVE 3. To determine the effects of AsA supplementation to the IVM, IVF and IVC media, on the maturation, fertilization and embryonic developmental parameters, and to assess the effects of adding AsA to vitrification and warming defined media on the vitrification survival of IVP-porcine blastocysts (Article 3).

Experiment 1. The effects of AsA supplementation in IVM, IVF and IVC media on maturation, fertilization and embryo development.

Experimental design

Oocyte maturation, fertilization, and embryo culture were performed in the presence or absence of 50 µg/mL of AsA in all possible combinations, which involved a total of 8 experimental groups. A total of 2744 oocytes were used in six replicates. A random subset of oocytes (n=149) and presumed zygotes (n=1142) from each group was fixed and stained at 44 h of IVM and 18 h after IVF to evaluate the maturation and fertilization parameters, respectively. The remaining presumptive zygotes (n=1602) were cultured to assess *in vitro* embryo development. Day 7 blastocysts were fixed and stained to assess their total cell number.

Results

The addition of AsA had no effect on the percentage of MII oocytes at 44 hours of maturation between treatment (81.4%) and control (83.5%). Supplementation of IVM, IVF and IVC media had neither any effects on fertilization parameters. Rate of sperm penetration was close to 70% with monospermy around 60% in all groups. The overall IVF efficiency of the IVP system ranged from 37.6 ± 8.5% to 47.3 ± 13.9% with no differences between treatment and control groups. The addition of AsA did not affect the development to 2-4 cells stage nor of blastocyst formation at the end of the culture period. The total efficiency of blastocysts production was around 30% in all cases. The quality of the *in vitro*-produced blastocysts in terms of total cell number (range from 44.1 ± 20.4 to 53.0 ± 26.2 cells) did not vary with the AsA supplementation.

Experiment 2. The effect of adding AsA to vitrification-warming media on the post-warming survival and quality of *in vitro*-produced porcine blastocyst.

Experimental design

The IVP of blastocysts was performed without AsA. Day-6 in IVP blastocysts (n=588) from 6 replicates were randomly divided into one of two groups, where vitrification and warming media were supplemented with 50 µg/mL of AsA (VW+ group) or not supplemented (VW- control). This AsA concentration was selected based on previous experiments (Kere et al., 2013). After warming, VW+ (n=281) and VW-control (n=307) blastocysts were cultured *in vitro* for 24 hours to assess the embryo survival and hatching rates. A random subset (n=21) of vitrified-warmed blastocysts classified as viable from each group were subjected to differential staining to assess the total number of cells, the number of cell in the ICM and the number of cells in the TE. Finally, the intracellular GSH and ROS levels from VW+, VW-control and some fresh *in vitro*-produced blastocysts (Fresh control) were measured.

Results

The addition of AsA during vitrification and warming enhanced ($P < 0.05$) blastocyst survival rate compared with VW-control embryos (51.1 ± 20.9% and 34.8 ± 21.4%, respectively). Total cell numbers and the distribution of cells between the TP and the ICM were also comparable between both vitrification groups. The vitrification and warming procedures increased ($P < 0.05$) intracellular ROS and decreased ($P < 0.05$) GSH levels, compared to controls. The addition of AsA to the vitrification-warming media decreased ($P < 0.05$) ROS production, but did not affect GSH. Those embryos vitrified and warmed without AsA (VW-control) displayed the highest ($P < 0.05$) intracellular ROS values, while those treated with AsA had intermediate ROS levels.

OBJECTIVE 4. To investigate the effects of parity, season and WEI on the reproductive and embryonic parameters at day 6 after insemination of donor sows superovulated at postweaning estrus (Article 4).

Experimental design

The experiment was conducted over a 3-year period in 18 trials using a total of 221 Duroc donor sows, with a lactation length of 21.8 ± 0.1 days. In each trial, only sows with a WEI of 3 to 4 days were selected as donors. Donor sows were selected at weaning from March 20 to June 20 (spring), from September 23 to December 20 (fall) and from December 21 to March 19 (winter), with two trials per season and per year. The selection of donor sows was also based on their reproductive history (average fertility and litter sizes > 90% and 10 piglets,

respectively), body condition (body condition score 2.75 to 3.25 on a five-point scale on the day of weaning) and parity (from 2 to 7).

Results

Of all superovulated donor sows (n=221), 82.8% (n=183) showed estrus at days 3 or 4 after weaning (42.1% and 40.7%, respectively). The rest of the sows (17.2%) showed estrus after day 4 of weaning and were not used in the study. Of 183 donor sows, 179 (97.8%) had embryos on day 6 post-insemination, and just 4 (2.2%) had only oocytes after flushing. The proportion of donor sows with ovarian cysts was 23.0%, with an average of 3.5 ± 0.2 cysts per sow. The mean ovulation rate was 23.8 ± 0.4 corpus luteum (ranging from 6 to 40 corpus luteum, CV=24.0%). The recovery and fertilization rates were 90.8 ± 0.8% and 96.3 ± 0.9%, respectively, and the mean number of viable embryos, oocytes or degenerate embryos obtained in the pregnant sows was 20.8 ± 0.4 and 1.0 ± 0.1, respectively. The proportion of transferable embryos in relation to the number of viable embryos was 94.3 ± 1.4%. The total number of transferable embryos collected from the inseminated donor sows (n=183) was 3508. There were no significant effects of parity, season, WEI or their interactions on pregnancy, number of cysts in sows with cysts or examined embryo parameters.

Related to the risk estimates of season, parity and WEI on the development of ovarian cysts in embryo donor sows, the multivariable logistic regression analysis showed that parity (range: 16.1% - 26.6%) and WEI (20.0% from sows with 4 days WEI and 25.8% from sows with 3 days WEI) were not associated with ovarian cyst rates. By contrast, season was significantly associated with ovarian cysts with a 3.5- to 5.5- fold increased risk of having cysts on ovaries in fall (OR=3.69, 95% CI 1.46 to 9.37) and winter (OR=5.57, 95% CI 2.00 to 15.51), respectively, compared to spring.

OBJETIVE 5. To explore the possibility of re-vitrify *in vivo*-derived porcine embryos (morulae and unhatched blastocysts) (Article 5).

Experiment 1. Evaluation of the effects of the re-vitrification of warmed porcine in vivo-derived embryos on the *in vitro* embryo survival.

Experimental design

Collected embryos (n=255) were assigned to one of two groups: a group that underwent a single vitrification and warming (VW, vitrification control group) and a fresh embryo control group. Each group was thereafter divided into two sub-groups according to

their embryonic developmental stage (morula or blastocyst). Embryos from the fresh control group were not vitrified, and embryo viability was evaluated at 24 hours of *in vitro* culturing. Viability and hatching rates of embryos from the VW group were evaluated at 24 hours post-warming. Subsequently, unhatched viable blastocysts were re-vitrified and re-warmed (RVW experimental group; n=136) and the viability of those embryos was assessed at 24 hours of *in vitro* culturing. Finally, we evaluated hatching rates of RVW and fresh control embryos at 48 hours of *in vitro* culturing.

Results

A high proportion (> 85%) of embryos survived the first vitrification and warming and achieved comparable survival rates to those of their fresh counterparts. In contrast, a subsequent second vitrification and warming was harmful to the embryos since their survival rates (63.9% for morulae and 60.0% for blastocysts) were significantly decreased ($P < 0.01$), when compared with those of the control groups. At 48 hours of culturing, hatching rates were similar in re-vitrified blastocysts derived from vitrified morulae and fresh control groups (50.8% and 55.3%, respectively). Significant differences ($P < 0.01$) in hatching rates were recorded in re-vitrified blastocysts derived from vitrified blastocysts and fresh control blastocysts (14.7% and 90.0%, respectively).

OBJECTIVE 6. To assess if the DSs is adequate for maintaining the viability and quality of vitrified *in vivo*-derived porcine embryos (morulae and unhatched blastocysts) for a 3-day storage period (Article 5).

Experiment 2. Evaluation of the efficacy of a DS dewar in maintaining the viability of vitrified embryos for a 3-day storage period

Experimental design

After vitrification, SOPS straws containing the embryos were stored in a LN_2 tank for one month. Then, the straws were transferred from the LN_2 tank to the DS (DS group; n=102) or to another LN_2 tank (control group; n=101) for an additional three days. In both groups, the embryos were divided according to their developmental stage (morula or blastocyst). After warming, embryos from the DS and the control groups were cultured for 24 hours to assess embryo survival and hatching rates. Finally, an aliquot of embryos from each group was subjected to a TUNEL assay to evaluate nuclei with DNA degradation and the apoptotic index.

Results

Embryos from the DS group presented similar survival rates to those of the LN_2 control group (range: 88-100%), regardless of the embryonic stage tested. The storage of morulae and blastocysts in DS did not affect embryo development towards the end of culture compared to the control group. The pattern of the frequency distribution of embryonic developmental stages was comparable between the groups. Although there were no differences in the total cell number of the resulting blastocysts between the DS and LN_2 groups (range: 119.9 ± 60.6 - 163.1 ± 39.2), the apoptotic index was affected, being slightly higher ($P < 0.05$) in those morulae and blastocysts stored in the DS (3.0 ± 2.6 y 2.6 ± 2.6, respectively), compared with LN_2 group (1.3 ± 1.8 y 1.7 ± 1.9, respectively) regardless of the developmental stage considered.

OBJETIVE 6. To investigate the effects of the recipients' parity on their reproductive performance after NsDU-ET (Article 6).

Experimental design

A total of 158 donors were selected based on their reproductive history (average fertility and litter sizes > 90% and 10 piglets, respectively). The weaned recipients (n=120) were selected based on their reproductive history (previous fertility > 90%; previous litter sizes > 9 piglets) and body condition (body condition score 2.75 to 3.25 on a five-point scale on the day of weaning) and grouped into three categories according to their parity: primiparous sows, sows of parity 2 and sows of parities from 3 to 5. Thirty transferable embryos (morulae and unhatched blastocysts) were non-surgically transferred into one uterine horn of each recipient.

The same operator performed all transfers. The experiment was comprised of a total of seven trials. Each trial was conducted in separate sessions over a 1-year period and consisted of 20-25 donors and 14-17 transfers. Within each trial, the donors were inseminated with sperm doses from the same boar, the embryos from each donor were equally and randomly allocated to each of the recipient groups, and a similar number of recipients from the three groups were included. The difficulties during the insertion of the NsDU-ET catheter through the cervix and uterine horn were evaluated according to the resistance felt by the operator while manipulating the catheter and grouped in three classes (1: no or light resistance, 2: moderate or severe resistance, 3: impossible to insert through the cervix). The correct position of the NsDU-ET catheter in the uterine horn was considered when no bends or kinks were observed in the catheter after removal (Martinez et al. 2004). The presence of

blood on the catheter during or after insertions and the incidence of vaginal discharges after transfers were recorded.

At 12 days after NsDU-ET, the recipients were evaluated daily for signs of oestrus. Pregnancy was diagnosed by ultrasonography on Days 20-22 post-ET. All pregnant sows were allowed to carry their litters to term, and the return to oestrus rate (regular and irregular), abortion rates, farrowing rates and litter sizes were recorded. The piglet production efficiency was calculated as the ratio of the number of total born piglets to the number of embryos transferred to all recipients.

Results

Of the 158 donors, 148 (93.7%) had embryos on Day 6 post-insemination, 9 (5.7%) had only oocytes after flushing and 1 (0.6%) had polycystic ovaries with no corpora lutea on her ovaries. The mean ovulation rate was 25.7 ± 5.0 corpora lutea (range 15 to 61 corpora lutea, CV=19.4%). The recovery rate was 97.0%, and the mean number of viable embryos, and oocytes and/or degenerate embryos obtained in the pregnant sows was 23.0 ± 3.1 and 1.8 ± 1.8, respectively (92.8% fertilization rate). The proportion of transferable embryos (compacted morulae and unhatched blastocysts) in relation to the number of viable embryos was 98.4%.

The non-transferable viable embryos were exclusively related to the presence of uncompacted morulae and hatched blastocysts. The total number of transferable embryos collected from the inseminated donors (n=158) was 3364, resulting in a donor to recipient ratio of 1.4 : 1.

The difficulties encountered during the insertion of the NsDU-ET catheter were considered as none or minor in 90.0% of the transfers. The parity [1(n=41), 2 (n=43) and 3 to 5 (n=36)] had no influence on the difficulty grade of the insertions, being more than 80 % of class 1, less than 15 % of class 2 and less than 5% of class 3. Two of 120 (1.7%) recipients were excluded from the study because it was not feasible to insert the catheter through the cervical canal. The catheter was appropriately inserted into a uterine horn in 111 of the 118 (94.1 %) remaining recipients, with no differences among the parity groups. Additionally, no perforations of the cervix or uterine wall were suspected during catheter insertions, the females' behaviour during the procedure was classified as good (no reaction or minor reaction) in all cases, and no vaginal discharges were observed after the procedure in any of the recipients. Regarding with the reproductive performance of the recipients with an appropriate insertion, the number of evaluated recipients were: 38 from parity 1, 40 from partiy 2 and 33 from parity 3 to 5. There were no differences in the pregnancy rates (range: 73.7% - 75.8%), farrowing rates (range: 68.4% - 72.7%), or litter sizes (range: 9.5 ± 3.3 - 9.8 ± 3.4) between

groups. Also, there were no differences between groups with respect to the piglets' birth weights (range: 1.5 ± 0.3 -1.7 ± 0.3) or piglet production efficiency (range: 22.3% - 23.0%).

CONCLUSIONS

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1. In the absence of vitrification, the toxic effects of the vitrification media on the GV oocytes were minimal and similar for both CPA combinations. The oocytes classified as viable after CPA exposure and IVM exhibited similar fertilization rates and embryo development capacity as control oocytes.

2. High-quality blastocysts can be produced from SOPS-vitrified immature oocytes. However, the blastocyst rate remained very low, and the developmental competence of the vitrified oocytes was reduced compared with the non-vitrified controls.

3. The interaction of Gns with the three MINs tested (dbcAMP, cycloheximide and cilostamide) accelerated meiotic progression to the MII stage. The presence of dbcAMP during the first period maturation increased or even doubled the capacity for oocyte development to the blastocyst stage.

4. Under our experimental conditions, the supplementation of IVM/IVF/IVC media with AsA at a concentration of 50 µg/mL failed to improve the IVP-outcomes. In contrast, the addition of AsA to chemically defined vitrification and warming media enhanced the blastocysts survival and decreased their ROS production.

5. The parity of donor multiparous sows did not affect the pregnancy and fertilization rates and the number and quality of 6-day-old embryos, regardless of the time of the year (from fall to spring) or the WEI (3 or 4 days).

6. Porcine blastocysts derived from vitrified and warmed morulae and blastocysts could be re-vitrified with quite good survival rates.

7. The DS is an efficient system for the storage of vitrified morulae and blastocysts for a storage period of three days, without affecting their viability after warming.

8. The pregnancy and farrowing rates and litter sizes were not affected by the recipients' parity after NsDU-ET.

ABBREVIATIONS

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AsA: Ascorbic acid

BSA: Bovine serum albumin

CI: Confidence interval

CPAs: Cryoprotectans

COCs: Cumulus oocytes complexes

DbcAMP: diburyryl cyclic adenosine monophosphate

DMSO: dimethyl sulfoxide

DPBS: Dulbecco´s phosphate-buffered saline medium

DSs: Dry shippers

ET: Embryo transfer

FDA: Fluorescein diacetate

Gns: Gonadotrophins

GV: Germinal vesicle

ICM: Inner Cell Mass

IVC: *In vitro* culture

IVF: *In vitro* fertilization

IVM: *In vitro* maturation

IVP: *In vitro* production

LN_2: Liquid nitrogen

MII: Metaphase II

MINs: Meiotic inhibitors

mTBM: Modified Tris-buffered medium

NCSU: North Caroline State University medium

NsDU: Non-surgical deep uterine

EG: Etylene glycol

OR: Odd ratios

PG: Propylene glycol

ROS: Reactive oxygen species

SD: Standard deviation

SEM: Standard error of the mean

SOPS: Super Open pull straw, Open pull straw

TE: trophectorderm

TL-HEPES-PVA: Tyrode´s lactate-HEPES PVA

V1: First vitrification medium

V2: Second vitrification medium

WEI: weaning estrus interval

RESUMEN GENERAL

INTRODUCCIÓN

Las tecnologías reproductivas porcinas han progresado significativamente en los últimos años ofreciendo importantes aplicaciones para la industria del sector porcino y también para la investigación. La implementación de protocolos de conservación y de transferencia de embriones podrían permitir el intercambio de material genético con un riesgo mínimo de transmisión de enfermedades y con costes reducidos, lo que podría ser de gran interés en el sector. La especie porcina no es solo importante a nivel de producción ganadera sino que también tiene gran relevancia en biomedicina, gracias a sus características fisiológicas similares con la especie humana (Rogers et al., 2008; Chorro et al., 2009; Vilahur et al., 2011). En este sentido, la especie porcina ha resultado ser el mejor modelo biomédico (Roura et al., 2016) y ha sido candidato como potencial donante o productor de tejidos y órganos humanos. Por estas razones, conseguir una mejora en los sistemas de producción *in vitro* (PIV) e *in vivo* de embriones porcinos más eficientes para la conservación y transferencia de embriones en esta especie podría tener un gran impacto tanto en la producción animal como en investigaciones biomédicas.

La PIV de embriones porcinos ha presentado numerosos desafíos en las últimas décadas (revisado de Abeydeera et al., 2002, revisado de Gil et al., 2010, revisado de Grupen et al., 2014). Los primeros lechones nacidos a partir de embriones producidos *in vitro* fueron obtenidos por Mattioli et al., (1989). Desde entonces, ha habido numerosos intentos de mejorar las técnicas de PIV con un progreso relativamente exitoso en la maduración *in vitro* (MIV), la fecundación *in vitro* (FIV) y el cultivo *in vitro* (CIV, revisado Gil et al., 2010). Sin embargo, a pesar de esta extensa investigación, existen diferentes impedimentos todavía sin resolver. Los sistemas de PIV actuales todavía tienen distintos fallos como: capacidad citoplasmática insuficiente de los ovocitos madurados *in vitro*, la fecundación polispérmica y las condiciones de cultivo subóptimas que conducen a una calidad deficiente de los embriones producidos *in vitro* y una baja eficiencia de la formación de blastocistos.

Desde un punto de vista práctico, para la PIV de embriones se requiere un gran número de ovocitos inmaduros. Estos ovocitos son generalmente recolectados de cerdas prepúberes sacrificadas en matadero. Sin embargo, no todos los laboratorios/centros de investigación tienen disponible un matadero cercano, por lo que la posibilidad de utilizar ovocitos criopreservados podría ser una alternativa al proporcionar más flexibilidad a los sistemas de PIV de embriones. La primera criopreservación exitosa de ovocitos porcinos fue lograda por Isachenko et al., (1998), y desde entonces, varias investigaciones se han centrado

en mejorar los protocolos de criopreservación para esta especie. En este aspecto, la vitrificación disminuye los daños ocasionados por las bajas temperaturas y parece ser el mejor método para la criopreservación de ovocitos porcinos. Aunque los ovocitos maduros son tradicionalmente preferidos para la vitrificación debido a que la supervivencia es relativamente alta, su fecundación y posterior desarrollo embrionario se ven seriamente comprometidos (Egerszegi et al., 2013; Somfai et al., 2012). Por el contrario, los complejos de células del cúmulos-ovocitos (CCOs) vitrificados en el estadío de vesícula germinal (VG) muestran bajas tasas de supervivencia, pero mantienen su capacidad para llevar a cabo una fecundación exitosa (Egerszege et al., 2013). Los crioprotectores (CPA) permeables más comúnmente usados para la criopreservación de ovocitos porcinos son: etilenglicol (EG), glicerol (Taniguchi et al., 2011), DMSO (Gupta et al., 2007), propilenglicol (PG, Somfai et al., 2014) y acetamida (Fuku et al., 1995). Entre ellos, el EG, que tiene alta permeabilidad y baja toxicidad, ha demostrado ser el CPA más eficaz para ovocitos porcinos (Wu et al., 2006; Huang et al., 2008). Aunque la combinación de EG y DMSO ha demostrado ser muy eficaz en la vitrificación de embriones porcinos (Cuello et al., 2008), el DMSO afecta negativamente a la competencia meiótica en el estadío de VG (Gupta et al., 2007) y ratón (Trounson et al., 1989) ovocitos. Estas investigaciones sugieren que el DMSO puede no ser adecuado para la vitrificación de ovocitos; Por lo tanto, el PG podría ser un sustituto apropiado para el DMSO debido a que es más permeable tanto en ovocitos porcinos como en embriones (Pedro et al., 2005). En este sentido, consideramos interesante la evaluación de la efectividad de la combinación de EG-DMSO y EG-PG para la vitrificación de ovocitos inmaduros porcinos.

La incidencia de polispermia es uno de los principales problemas en los protocolos de PIV de embriones porcinos actuales. , que todavía se mantiene en 30-50% en la mayoría de los laboratorios (Funahashi y Nagai., 2000; Gil et al., 2004; Suzuki et al., 2000; Martinez et al., 2017). Numerosos estudios (revisado de Aveydeera., 2002, revisado de Funahashi., 2003 y Kikuchi et al., 2002) se han llevado a cabo con el objetivo de identificar las posibles deficiencias de ovocitos o mejorar las condiciones de FIV. La exposición de los gametos, antes o durante la FIV, a células del oviducto (Kim et al., 1997; Nagai y Moore, 1990) o fluido folicular (Funahashi y Day., 1993), han demostrado algunos beneficios que mejoran la eficacia de la fecundación. Además, otros factores tales como el uso de espermatozoides congelados (Suzuki et al., 2000), la reducción del tiempo de co-cultivo de los gametos (Grupen y Nottle., 2000; Funahashi y Romar., 2004; Gil et al., 2004) o diversas modificaciones en el sistema utilizado para regular el número de espermatozoides penetrantes cerca de los ovocitos (Funahashi y Nagai., 2000; Wheeler et al., 2004) también han sido evaluados. Sin embargo, aunque estas técnicas han

logrado moderar la frecuencia de polispermia, el problema aún permanece. Un punto crítico de la PIV, el cual puede afectar a la polispermia y también al subsecuente desarrollo embrionario y calidad de los embriones, es la insuficiente maduración citoplasmática de los ovocitos *in vitro*. Se sabe que los ovocitos procedentes de los ovarios de cerdas prepúberes sacrificadas se encuentran en diferentes fases de crecimiento y etapas de desarrollo. Además, los ovocitos de mamífero pueden reiniciar la meiosis espontáneamente cuando se extraen del folículo (Pincus y Enzmann., 1935), dando como resultado ovocitos con una maduración nuclear y citoplasmática asincrónica. Esta asincronía nuclear-citoplasmática se ha considerado principalmente responsable de las diferencias en el desarrollo embrionario entre ovocitos porcinos madurados *in vitro* (Abeydeera., 2002). Para resolver este problema, es interesante el empleo de técnicas de sincronización. Estas técnicas se basan en el uso de inhibidores reversibles de la meiosis (IRMs) y se han utilizado en diferentes especies, incluida la especie humana (Lange Consiglio et al., 2010; Leal et al., 2012; Nogueira et al., 2006). En porcino, los IRMs específicos tales como el inhibidor de la síntesis de proteínas cicloheximida (Le Beux y et al., 2003; Ye y et al., 2005; Ye et al., 2002), el monofosfato de adenosina cíclico dibutirílico (dbcAMP; Funahashi et al., 1997), el inhibidor de la quinasa dependiente del ciclo roscovitina (Romar y Funahashi, 2006) y los inhibidores de la fosfodiesterasa, como la cilostamida (Dieci et al., 2013; Laforest et al., 2005; Sasseville et al., 2006), han obtenido una inhibición completa y reversible de la meiosis. Además, la reanudación meiótica está controlada por gonadotropinas (Gns), por lo que la evaluación de las interacciones de los IRMs con las Gns podría ser interesante.

El último problema, pero no menos importante, está relacionado con las condiciones del CIV. A pesar de los avances en el CIV de los embriones porcinos, las condiciones de cultivo son subóptimas y la eficiencia es aún muy baja (revisado de Gil et al., 2010; Somfai et al., 2011). Los medios de cultivo más comúnmente utilizados son los medios de la Universidad del Estado de Carolina del Norte (NCSU): NCSU-23 y NCSU-37, que fueron descritos por Petters y Wells., (1993). Aunque gracias a estos medios, los embriones son capaces de llegar hasta el estadio de blastocisto se ha demostrado que estos medios no son adecuados ya que los embriones cultivados *in vitro* tienen un menor desarrollo y una menor calidad comparado con los embriones cultivados *in vivo*, independientemente de si ellos fueron producidos *in vitro* o *in vivo* (revisado de Kikuchi et al., 2004). Para mejorar los resultados de PIV es esencial estudiar e intentar imitar las condiciones de cultivo *in vivo*. Una de las principales diferencias entre las condiciones *in vitro* e *in vivo* está relacionada con el estrés oxidativo (Fisher y Bavister., 1993). El ambiente de la PIV de embriones tiene un contenido de oxígeno más alto que en

condiciones *in vivo*, lo que resulta en un aumento de la producción de sustancias oxígeno reactivas (ROS) (Luvoni et al., 1996; Takashi et al., 2002). Se ha demostrado que estos altos niveles de ROS durante la PIV de embriones son perjudiciales para gametos y embriones (Luberda., 2005; Silva et al., 2007; Kim et al., 2015). En consecuencia, la protección de los ovocitos y los embriones contra el estrés oxidativo durante el CIV es crucial para mejorar la eficiencia del proceso de PIV y la calidad de los embriones. Para superar este obstáculo, una estrategia comúnmente utilizada es la adición de antioxidantes a los diferentes medios de PIV (Tao et al., 2004; Funahashi., 2005; Nagai et al., 2006; revisado de Gajda., 2009; Kang et al., 2013). Dentro de los diferentes antioxidantes, el ácido ascórbico (AsA) es uno que brinda mayores expectativas. El AsA es el antioxidante más importante en fluidos extracelulares (Warren et al., 2000) y se ha probado en embriones producidos *in vitro*, mostrando algunas propiedades beneficiosas. En porcino, el AsA ha mostrado efectos positivos en la maduración nuclear de los ovocitos (Tao et al., 2004) y la formación de blastocitos después de la activación partenogenética (Kere et al., 2013). La adición de AsA también parece proteger a los embriones contra el estrés oxidativo durante el CIV, mejorando la capacidad de desarrollo de los embriónes después de la FIV (Hossein et al., 2007) o la partenogénesis (Kere et al., 2013; Hu et al., 2012). En algunos estudios, la suplementación con AsA durante CIV también mejoró la calidad de los blastocistos en términos de números de células (Hu et al., 2012) o la supervivencia después de la vitrificación (Castillo-Martin et al., 2014). Sin embargo, no existe un estudio sistemático que investigue la influencia del AsA en cada paso del sistema de PIV de embriones y la presencia de posibles efectos sinérgicos.

La ineficiencia de la PIV de embriones nos conduce a emplear otras técnicas reproductivas tales como la obtención de embriones *in vivo*. Si bien esta técnica es compleja, costosa y lleva mucho tiempo, puede ser una alternativa a la PIV cuando se requiere un alto número de embriones de buena calidad. Un factor clave para obtener embriones numerosos y de buena calidad es la selección de las cerdas donantes, lo cual ha recibido poca atención en el campo de la transferencia de embriones (TE) en porcino. En general, además del grado genético, las donantes se seleccionan basándose en una buena condición corporal y un estado de salud adecuado. Sin embargo, se sabe que el número de partos influye en la fertilidad y prolificidad de las cerdas, con mayores tasas de partos y tamaños de camadas en multíparas que en primíparas. Otro factor esencial en el rendimiento reproductivo de las donantes es el intervalo destete-celo (IDC) debido a que tiene una relación inversa con la duración del estro y el momento de la ovulación (Soede y Kemp, 1997; Belstra et al., 2004). La mayoría de las cerdas destetadas muestran un estro fértil entre 3 y 5 días después del destete. Estas cerdas

con un IDC específico generalmente se agrupan y, por razones prácticas, los programas de TE usan estas cerdas como donantes. Estas diferencias en el IDC podrían influir en la duración del estro y en el momento de la ovulación. Como consecuencia, el número de embriones no transferibles podría aumentar debido a la presencia de mórulas pre-compactas (ovulaciones tardías) o blastocistos eclosionados (ovulaciones tempranas), que no son aconsejables para la TE debido a su poca eficiencia después de las transferencias o por razones sanitarias, respectivamente. Otro factor principal es la influencia de la estación en el rendimiento reproductivo de las cerdas. Comparado con otras épocas del año, en verano, es común un IDC más largo y una fertilidad más baja. Aunque también se ha observado una interacción del número de partos y la estación con las características del celo y la ovulación en cerdas destetadas (Knox y Rodríguez Zas., 2001), aún no se cuenta con información sobre cómo la temporada afecta la producción de embriones en las cerdas donantes. Estos aspectos sobre las donantes (número de partos previos, estación e IDC) fueron evaluados en esta tesis doctoral.

Independientemente de si los embriones se obtienen mediante técnicas *in vivo* o *in vitro*, el objetivo final de cualquier programa es la TE. La tecnología de la TE ha sido demandada por la industria porcina durante décadas debido a sus numerosas aplicaciones, particularmente para el intercambio seguro de material genético de alto valor con costes de transporte reducidos. Además, esta técnica disminuye considerablemente los problemas de bienestar animal. A pesar de estas ventajas, el uso comercial de la TE en porcino es aún muy limitado en comparación con otras especies (revisado de Martinez et al., 2016). Las razones principales para el uso limitado de la TE en porcino se deben a la necesidad de procedimientos quirúrgicos y las dificultades para criopreservar embriones porcinos. Sin embargo, en los últimos años, la situación ha cambiado gracias a la mejora de los procedimientos de vitrificación (Sánchez-Osorio et al., 2010) y al desarrollo tecnológico de un procedimiento para la TE intrauterina profunda no quirúrgica (NsDU) (revisada Martínez 2016).

En los programas de TE, los embriones deben almacenarse desde el momento de su recolección hasta que puedan transferirse a las receptoras. Hoy en día, el único método eficaz para la conservación a largo plazo de embriones porcinos es la vitrificación, que evita la formación de cristales de hielo en los espacios intracelular y extracelular (Fuller et al., 2004). La mejora de los procedimientos de vitrificación, específicamente mediante un sistema que consiste en una pajuela abierta (Super Open pull Straw: SOPS) (Vajta., 1997), ha proporcionado altas tasas de supervivencia de embriones post-calentamiento *in vitro* y tasas más altas de gestación y de partos después de la transferencia de embriones porcinos obtenidos *in vivo*, transferidos de forma quirúrgica y vitrificados sin ningún tratamiento previo (revisado

Martinez et al., 2013). Aunque se ha producido un progreso importante en la vitrificación de los embriones porcinos, existen muchos factores que todavía no se han resuelto. Entre estos factores, la re-vitrificación de embriones y el transporte de embriones por aire en fase de vapor de nitrógeno líquido (NL_2) son de especial interés. En algunas circunstancias durante la TE (por ejemplo, cuando el número de embriones calentados excede el número de embriones necesarios para ser transferidos a las receptoras, bien por problemas técnicos durante la inserción del catéter de transferencia no quirúrgica, bien por problemas innatos de la propia receptora) podría existir un exceso inesperado de embriones calentados. Aunque estos embriones calentados de manera extra podrían usarse para aumentar el número de embriones transferidos por receptora, lo ideal sería que pudieran volver a vitrificarse y almacenarse nuevamente para futuras TEs. En este sentido, la posibilidad de volver a vitrificar debería proporcionar más flexibilidad a los programas de TE. Sin embargo, los efectos de la vitrificación sobre las tasas de supervivencia de embriones post-calentamiento en cerdos aún no se ha evaluado. Por otro lado, el transporte aéreo de embriones en tanques de NL_2 está prohibido por la Asociación Internacional de Transporte Aéreo (IATA). Por lo tanto, la única opción para el transporte aéreo de embriones criopreservados es utilizar contenedores especiales. Los llamados tanques secos (DS). Los DS se han usado para semen humano criopreservado (Lim et al., 2010; Punatanasakchai et al., 2008) y ovocitos (Cobo et al., 2010) y para embriones de ratón vitrificados (AbdelHafez et al., 2011). Sin embargo, hasta donde sabemos, no existen estudios publicados sobre la efectividad de los DS para el transporte de embriones porcinos vitrificados.

Finalmente, es importante destacar el desarrollo de la NsDU-TE, un procedimiento simple, seguro, efectivo y práctico que se puede realizar en condiciones de campo sin instalaciones específicas. Gracias a las mejoras en el procedimiento de la NsDU-TE, se han logrado excelentes resultados reproductivos (Martínez et al., 2004, Angel et al., 2014a, b). Sin embargo, existen aún muchos factores que pueden afectar a los resultados finales de la TE en la especie porcina. Algunos de estos factores han sido estudiados ampliamente (Angel et al., 2014a, B., Martinez et al., 2014, 2015) tales como el efecto de la superovulación de las donantes; la sincronización entre la etapa de desarrollo del embrión y las receptoras; el uso de embriones almacenados a corto y largo plazo; y la cantidad de embriones transferidos por receptora. Sin embargo, hay otros factores que pueden afectar el éxito de la NsDU-TE, factores que deben ser evaluados. Uno de estos factores es la selección de las receptoras. En general, al igual que los donantes, esta selección se basa en una buena condición corporal y un excelente estado de salud. Sin embargo, hay otros factores que pueden desempeñar un papel clave en

los resultados de TE. Por ejemplo, se supone que las tasas de partos y tamaños de camadas de cerdas primíparas son usualmente más bajas que las cerdas multíparas y que el rendimiento reproductivo más alto aparentemente se alcanza desde el tercer parto (Koketsu et al., 1999; Hughes y Varley., 2003) hasta el sétimo parto (Flowers y Alhusen., 1992; Hughes, 1998). A pesar de estas observaciones, en todos los estudios publicados hasta la fecha sobre TE porcina, se han seleccionado de forma arbitraria cerdas nulíparas, primíparas o multíparas como cerdas receptoras (Berthelot et al., 2001; Brüssow et al., 2000; Dobrinsky et al., 2000; Cameron et al., 2004; Martínez et al., 2014, 2015; Beebe et al., 2005, 2011; Cuello et al., 2005; Nakazawa et al., 2008) y en ninguno de estos estudios se evaluó el efecto del número de partos de las receptoras sobre la fertilidad y prolificidad post-TE. El uso de cerdas multíparas como receptoras presenta diversas ventajas. Aunque el catéter de NsDU-TE se puede utilizar tanto en nulíparas como en multíparas (Martínez et al., 2004), los porcentajes de dificultad de inserción del catéter son menores en estas últimas. Asimismo, a menudo se prefiere el uso de cerdas multíparas como receptoras porque el destete es una forma excelente y natural para la sincronización del estro en cerdas (Britt et al., 1985). El número de estros previos de las cerdas nulíparas es crucial para obtener un éxito adecuado de las inserciones, lo cual exige un control exhaustivo de los ciclos en este tipo de cerdas (Cuello et al., 2005).

OBJETIVOS

Los principales objetivos de la presente tesis Doctoral fueron incrementar la eficiencia de los sistemas actuales de PIV de embriones así como la mejora de las técnicas de transferencia de embriones. Con este propósito, los objetivos específicos contenidos en estas tesis fueron:

1. Analizar la eficacia de dos combinaciones de CPAs: EG+DMSO y EG+PG para la vitrificación de ovocitos en estadio de VG sobre los parámetros de viabilidad, fecundación y desarrollo embrionario. (Artículo 1).

2. Evaluar el efecto de tres IRMs, dbcAMP, cicloheximida y cilostamida, y sus interacciones con las Gns sobre la maduración meiótica y el desarrollo embrionario de ovocitos porcinos. (Artículo 2).

3. Determinar los efectos de la adición de AsA, en los medios de maduración, fecundación y cultivo embrionario sobre los parámetros de maduración, fecundación y desarrollo embrionario, así como el efecto de la adición de dicho antioxidante en los medios de vitrificación sobre la viabilidad embrionaria post-calentamiento (Artículo 3).

4. Investigar los efectos del número de partos, la estación del año y el intervalo destete-celo sobre los parámetros embrionarios de cerdas donantes superovuladas en el celo post-destete (Artículo 4).

5. Explorar la posibilidad de re-vitrificar embriones porcinos obtenidos *in vivo* (morulas y blastocistos no eclosionados). (Artículo 5).

6. Evaluar la eficacia de los tanques de nitrógeno seco para mantener la viabilidad y la calidad de embriones porcinos obtenidos *in vivo* (morulas y blastocistos no eclosionados) y vitrificados durante un periodo de 3 días. (Artículo 5).

7. Investigar en cerdas receptoras cómo influye el número de partos previos en su capacidad reproductiva después de la NsDU-TE. (Artículo 6).

MATERIAL Y MÉTODOS

Todos los procedimientos experimentales se han llevado a cabo según la directiva de 2010/63/EU EEC para animales de experimentación y fueron revisados y aprobados con antelación por el Comité Ético de Experimentación Animal de la Universidad de Murcia, España (Código: 183/2015).

Reactivos

Todos los reactivos químicos y medios utilizados en este trabajo fueron de la marca Sigma-Aldrich Química S.A. (Alcobendas, España) a menos que se indique lo contrario.

Medios

El medio de transporte de los ovarios desde el matadero al laboratorio fue una solución de NaCl al 0’9% (v:v) con kanamicina 70 µg/mL. El medio utilizado para recolectar los complejos cumulus ovocitos (CCOs) fue el lactato de Tyrode- HEPES suplementado con 10 mM de HEPES y 0.1% (w:v) de polinil-alcohol (TL-HEPES-PVA; Funahashi et al., 2000; Martinez et al., 2014). El medio de maduración de los ovocitos fue TCM-199 (Gibco Life Technologies SA, Barcelona, España). El medio básico utilizado para FIV fue el medio tamponado Tris modificado (Abeydeera and Day, 1997), suplementado con 2’0 mM de cafeína y 0’2% de albúmina sérica bovina (BSA). El medio utilizado para el lavado de los espermatozoides fue la solución salina con tampón fosfato de Dulbecco (PBSD; Gibco, Grand Island, NY) al que se le añadió 4 mg/mL de BSA. El medio de cultivo de embriones fue el North Carolina State University 23 (NCSU23; Petters y Wells 1993) suplementado con BSA al 0’4% (p/v) y un 10% de suero fetal bovino en el momento de su utilización. El medio de recogida de los embriones y el medio base de vitrificación y calentamiento fue TL-HEPES-PVA. Para la preparación de los medios de vitrificación (V1 y V2) el medio base se suplementó con un 7’5% de dimetilsulfóxido (DMSO) y con un 7’5% de etilenglicol (EG) para la preparación del V1 y con un 16% de DMSO, un 16 % de EG y 0’4 M de sacarosa para la preparación del V2. El medio de calentamiento se preparó con TL-HEPES-PVA suplementado con 0’13 M de sacarosa. El medio de recolección de embriones y transferencia de embriones fue TL-HEPES-PVA.

Condiciones de incubación ovocitos/embriones

Al menos que otra cosa sea indicada, los ovocitos/embriones se incubaron a 38’5 °C con un 5% de CO_2 en aire y 95%-97% de humedad relativa.

Recogida de los ovocitos y MIV

Los ovarios se obtuvieron a partir de cerdas prepúberes en un matadero local. Los CCOs fueron recolectados con una hoja de bisturí, a partir de los folículos de tamaño mediano (3-6 mm de diámetro) en TL-HEPES-PVA. Los CCOs rodeados por una masa compacta de células del cúmulus se seleccionaron y se lavaron en medio de maduración. A continuación, se colocaron en grupos de 75-80 CCOs en placas de cuatro pocillos con medio de maduración suplementado con hormonas (eCG y hCG) y se incubaron durante 22 horas. Los ovocitos se incubaron posteriormente durante otras 20-22 horas en medio de maduración sin hormonas.

Fecundación *in vitro*

Después de la MIV, las células del cúmulus se retiraron con 0’1% de hialuronidasa diluida en medio de maduración, mediante vortex durante 2 min. Los ovocitos denudados se lavaron en medio de MIV y en medio de FIV y se colocaron en grupos de 30 (gotas de 50 µL) en placas Petri con medio de FIV. Los ovocitos se mantuvieron en la incubadora hasta que se añadieron los espermatozoides. Para la FIV se utilizó semen criopreservado y calentado procedente de un cerdo adulto. Después de la descongelación, se lavaron 100 µL de semen en PBSD. El pellet resultante se resuspendió en medio de FIV ajustando la concentración, de modo que al añadir 50 µL de la suspensión espermática final se consiguiera un ratio de espermatozoides: ovocito de 1000:1. Finalmente, los gametos se coincubaron durante 5 horas.

Cultivo *in vitro* de embriones

Después de la coincubación de gametos, los presuntos cigotos se lavaron en medio de CIV para retirar los espermatozoides que no estaban unidos a la zona pelúcida. Los presuntos cigotos (30 cigotos por pocillo) fueron colocados en una placa de cuatro pocillos con medio de cultivo de embriones sin glucosa, suplementado con 0’3 mM de piruvato y 4’5 mM de lactato durante 2 días. Luego se cambió a un medio de cultivo de embriones con 5’5 mM de glucosa, donde permanecieron durante 5 días adicionales.

Evaluación de los parámetros de maduración, fecundación y desarrollo embrionario.

Para evaluar los parámetros de maduración y fecundación, los presuntos cigotos se fijaron a las 44 horas después de la maduración y a las 18 horas después de la fecundación y se tiñeron con 1% de lacmoid. Los ovocitos con una cromatina rodeada por una membrana nuclear o los ovocitos con una cromatina condensada pero sin la extrusión del cuerpo polar fueron clasificados como inmaduros. Los ovocitos se consideraron maduros cuando sus cromosomas estaban en metafase y tenían un cuerpo polar extruido (MII). Los presuntos cigotos se consideraron penetrados cuando contenían una o más cabezas de espermatozoide hinchadas y/o pronúcleos masculinos y dos cuerpos polares. La tasa de penetración fue considerada como el ratio del número de espermatozoides penetrados sobre el total de ovocitos inseminados. La tasa de monospermia fue considerada como el ratio de ovocitos con un pronúcleo masculino, un pronúcleo femenino y dos cuerpos polares sobre el total de ovocitos penetrados. La eficiencia de la fecundación fue el ratio del número de ovocitos monospérmicos sobre el total de ovocitos inseminados.

La tasa de división se determinó al día 2 de cultivo y se definió como el porcentaje de presuntos cigotos divididos en dos y cuatro células sobre el porcentaje total de presuntos cigotos cultivados. La formación de blastocitos se determinó el día 7 y se definió como el porcentaje de embriones de 2-4 células que se desarrollaron hasta la etapa de blastocisto. La eficacia total se consideró como el porcentaje del número total de presuntos cigotos cultivados que alcanzaron la etapa de blastocisto. Para evaluar el número total de células, algunos blastocistos se fijaron en paraformaldehído al 4% en PBS. Los embriones se lavaron con PBS y se colocaron en un portaobjetos en gotas de 4 µL de VECTASHIELD (Vector Labs, Burlingame, CA, EE.UU.) que contenía Hoechst 33342 10 µg/mL. Por último, se contó el número total de núcleos que mostraron fluorescencia azul.

Vitrificación y calentamiento de embriones u ovocitos

La vitrificación de los embriones/ovocitos se realizó según el método descrito por Cuello et al., (2008). Brevemente, los embriones/ovocitos, en grupos de 5 a 6, se lavaron en TL-HEPES-PVA y se equilibraron en el primer (V1) y segundo (V2) medio de vitrificación durante 3 y 1 minutos, respectivamente. Durante el último equilibrado, los embriones/ovocitos se colocaron en una gota de 1 µL y se cargaron en el extremo delgado de una pajuela SOPS (Minitüb, Tiefenbach, Alemania) por capilaridad. Posteriormente, la pajuela se sumergió en NL_2. Después del almacenamiento en NL_2, los embriones/ovocitos se calentaron mediante el método de calentamiento directo (Cuello et al., 2004). Brevemente, las pajuelas fueron sumergidas verticalmente en un pocillo con medio de calentamiento. Los embriones se

equilibraron en este pocillo durante 5 minutos. Finalmente, los embriones se lavaron en TL-HEPES-PVA y se cultivaron en medio de MIV o CIV.

Evaluación de la viabilidad de los ovocitos

La viabilidad de los ovocitos se evaluó usando tinción con diacetato de fluoresceína (FDA) como se describe por Mohr y Trounson., (1980). La solución de trabajo se preparó mediante una dilución 1:2000 de la solución madre de la FDA en DPBS para alcanzar una concentración final de 2’5 mg/mL de FDA. Los CCOs se tiñeron en 500 mL de esta solución durante 2 minutos a 37 °C en una habitación oscura, y finalmente, se lavaron tres veces en DPBS. Los ovocitos se evaluaron con irradiación ultravioleta usando un filtro GFP-II bajo un microscopio de fluorescencia invertido. Los ovocitos vivos exhibieron fluorescencia verde brillante, y la viabilidad FDA se calculó como el porcentaje de ovocitos vivos del número total de ovocitos evaluados.

Evaluación de los embriones después del calentamiento.

Las mórulas que progresaron al estadio de blastocisto durante el CIV y los blastocistos que reformaron el blastocele con apariencia buena o excelente, fueron considerados viables. La tasa de supervivencia fue determinada como el ratio de embriones viables a las 24 horas de CIV respecto al total de embriones cultivados.

Tinción diferencial

El número de células procedentes de la masa celular interna (MCI) o del trofoectodermo fue determinada usando un protocolo de inmunofluorescencia basado en el descrito por Wydooghe et al., (2001). Brevemente, los embriones fueron fijados con un 4% de paraformaldehido y permeabilizados. Posteriormente, los embriones fueron incubados en una solución de 2 N HCL durante 20 minutos y luego en una solución de 100 mM Tris-HCL (pH8.5) durante 10 minutos. Después de lavarlos, los embriones se incubaron durante 6 horas en una solución bloqueante. A continuación, los blastocistos fueron incubados con el anticuerpo primario CDX-2 (1:200; BioGenex, CA, USA) y con donkey anti-mouse IgG-Alexa Fluor® 568 conjugate (1:1000 in blocking solution; Invitrogen, Rockford, USA). Finalmente, los embriones se colocaron en gotas de 4 µL de Vectashield (Vector Labs, Burlingame, CA, USA) con 10 µg/mL de Hoechst 33342 y se contabilizó tanto el número total de células (fluorescencia azul) como el número de células del trofoectodermo (fluorescencia roja).

Determinación de los niveles de GSH y ROS

Los niveles de GSH y ROS intracelular se determinaron mediante CellTraker Blue (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; CMF2HC; Invitrogen, ThermoFisher scientific, Massachusetts, EEUU) y H2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate; Invitrogen), respectivamente. Los embriones se incubaron en 10 µM de CellTraker Blue y 10 µM de H2DCFDA (2'.7'-diacetato de diclorodihidrofluoresceina). La fluorescencia se observó inmediatamente bajo un microscopio de fluorescencia con filtros ultravioleta (370 nm para GSH y 460 nm para ROS). La intensidad de fluorescencia de cada embrión se analizó mediante el programa Image J (versión 1.5h; National Institutes of Health, Bethesda, MD, EEUU).

Evaluación de la apoptosis celular

Los niveles de apoptosis se determinaron mediante la técnica TUNEL descrita por Byrne et al., (1999) y Brison y Schultz (1997). Para ello se empleó el kit comercial de detección de apoptosis celular APO-BrdUTM TUNEL Assay Kit (A23210; Invitrogen, Oregon, USA). Brevemente, los embriones se fijaron con un 4 % de paraformaldehido y se permeabilizaron. El control positivo fue incubado con ADNasa. Los embriones de ambos grupos se lavaron y se incubaron en gotas que contenían una enzima terminal transferasa; Bromodioxiuridina trifosfato (Br-dUTP) y anticuerpos específicos de Br-UTP marcados con fluoresceína. Por último, bajo un microscopio de fluorescencia, se contaron los núcleos que mostraron fluorescencia verde (células apoptóticas TUNEL positivas). El índice de apoptosis se calculó como el porcentaje de núcleos positivos a TUNEL con respecto al total de núcleos teñidos con Hoechst.

Donantes y receptoras de embriones

Se utilizaron cerdas (desde 2 a 6 partos) con un peso normal y buen estado de salud. Las cerdas se alojaron en jaulas individuales en una nave con ventilación controlada. Las cerdas recibieron una ración comercial de acuerdo a sus necesidades nutricionales y disponían de agua *ad libitum*. Para los eyaculados, se utilizaron cerdos adultos procedentes de un centro de inseminación artificial (AIM Iberica, Murcia, Spain).

Detección del estro, inseminación artificial, obtención y evaluación de los embriones

La detección del estro se realizó dos veces al día comenzando el día siguiente del destete con la ayuda de un verraco y ejerciendo presión en la parte posterior del dorso. Las cerdas en estro fueron inseminadas con dosis de inseminación previamente preparadas. La recogida de los embriones se realizó vía quirúrgica mediante una laparotomía el día 6 del ciclo,

considerándose el día 0 el día del inicio del estro. Las cerdas fueron previamente sedadas con azaperona (Stresnil®, Landegger Strasse, Austria; 2 mg/kg body weight, i.m.). La anestesia general se indujo mediante tiopental sódico (B. Braun VetCare SA, Barcelona, España; 7 mg/kg body weight, i.v.) y fue mantenida con isoflurano (IsoFlo®, Madrid, España). Una vez exteriorizado el útero y los ovarios, se procedió al recuento de los cuerpos lúteos. Los embriones fueron recogidos mediante el método descrito por Martinez et al., 2014 mediante el lavado de cada cuerno uterino con 30 mL de TL-HEPES-PVA. Los embriones fueron evaluados mediante un esteromicroscopio. Aquellas estructuras de una célula y los embriones con un desarrollo inadecuado para el día 6 de gestación se consideraron como ovocitos no fecundados o embriones degenerados, respectivamente. Los embriones en estadio de mórula o blastocisto no eclosionados y con una morfología buena o excelente según los criterios establecidos por la sociedad internacional de transferencia de embriones (IETS; Wright et al., 1998) fueron considerados viables y aptos para las experiencias.

Transferencia embrionaria uterina profunda no quirúrgica

Las NsDU-TE se realizaron en cerdas receptoras sin tratamiento hormonal utilizando el método descrito anteriormente por Angel et al., (2014a). Brevemente, antes de la transferencia, cada receptora recibió una sola inyección intramuscular de una suspensión de amoxicilina de acción prolongada (Clamoxyl® LA; Pfizer, Madrid, España) a una dosis de 15 mg/kg. Las receptoras fueron alojadas en jaulas de gestación en una pequeña habitación exclusivamente utilizada para ese fin. El área perineal de las receptoras se limpió a fondo, la vulva se lavó y se descontaminó con clorhexidina. Para la NsDU-TE se usaron catéteres de transferencia no quirúrgica (Deep Blue ET catéter, Minitub, Tiefenbach, Alemania). Cuando el catéter se insertó completamente en un cuerno uterino, se conectó una jeringa de 1 mL al catéter, la cual contenía los embriones en 0’1 mL de medio TL-HEPES-PVA. Para expulsar los embriones del catéter hacia el útero se utilizó un volumen adicional de 0’3 mL de medio TL-HEPES-PVA.

Análisis estadístico

El análisis estadístico se realizó utilizando el paquete estadístico IBM SPSS 19 (SPSS, Chicago, IL, EE. UU.), y las diferencias se consideraron significativas con P<0’05. Los resultados se expresaron como la media ± DE (desviación estándar) o la media ± SEM (error estándar de la media). Los datos con porcentajes se compararon usando la prueba de Fisher. Las variables binarias (media ± DE) y las variables continuas se analizaron mediante la prueba de Kolmogorov-Smirnov para evaluar el supuesto de normalidad. Se compararon las medias de

más de dos grupos utilizando un análisis de varianza de modelo mixto (ANOVA), seguido de la prueba post hoc de Bonferroni. Las comparaciones por pares de medias se realizaron usando la prueba t de Student. Los efectos de la estación, el número de partos y el IDC se evaluaron utilizando modelos lineales mixtos. El análisis de regresión logística multivariable con la presencia o ausencia de quistes en los ovarios como variable dependiente se utilizó para evaluar los odds ratios (ORs) y los correspondientes intervalos de confianza (ICs) del 95% para las variables independientes de número de partos, estación y IDC.

DISEÑO EXPERIMENTAL, ANÁLISIS ESTADÍSTICO Y RESULTADOS

OBJETIVO 1: Analizar la eficacia de dos combinaciones de CPAs, EG+DMSO y EG+PG, para la vitrificación de ovocitos en estadio de VG sobre los parámetros de viabilidad, fecundación y desarrollo embrionario. (Article 1).

Experimento 1. Evaluación de la viabilidad de los ovocitos porcinos después del equilibrado en EG+DMSO o EG+PG y subsecuente desarrollo embrionario.

Diseño experimental

En un total de tres replicados evaluamos la toxicidad de los medios de vitrificación, usando dos combinaciones de crioprotectores distintas (grupo EG + DMSO o grupo EG + PG). Los ovocitos (n=998) se sometieron a un equilibrado en los medios de vitrificación y calentamiento pero sin ser vitrificados. La viabilidad fue determinada mediante tinción FDA dos horas después del equilibrado y los ovocitos vivos fueron seleccionados para la MIV. A las 44 horas después de la MIV, los ovocitos con membrana citoplasmática lisada fueron considerados degenerados y fueron eliminados. A las 18 horas después de la FIV algunos presuntos cigotos fueron fijados para evaluar los parámetros de fecundación. El resto de los cigotos fueron cultivados *in vitro* para comparar los parámetros de desarrollo embrionario de los ovocitos expuestos a los CPAs con el grupo control.

Resultados

La viabilidad de los ovocitos 2 horas después del equilibrado en los medios con EG + DMSO y EG + PG fue muy elevada (95’7 ± 0’4% y 95’6 ± 1’1%, respectivamente) pero más baja ($P < 0'01$) que la observada en el grupo control (99’5 ± 0’8%). Mayores porcentajes ($P < 0'05$) de ovocitos vivos pertenencientes a los grupos EG + DMSO (18’1 ± 2’3%) o EG + PG (19’4 ± 2’6%) degeneraron a las 44 horas de maduración comparados con los del grupo control (7’6 ±

1’3%). A las 18 horas después de la FIV, el número de ovocitos inmaduros (< 12%) y degenerados (< 3%) fue similar para todos los grupos. No se observaron diferencias en la penetración (rango 74’5 ± 2’4% -77’5 ± 2’6%), monospermia (rango: 46’4 ± 4’1% - 57’5 ± 3’4%) y eficiencia (rango: 36’7 ± 3’0% - 49’2 ± 2’2%) entre grupos. La exposición de los ovocitos con los CPAs no afectó su capacidad de desarrollo en términos de divisiones (rango: 58’1 ± 3’3% - 61’6 ± 3’2%), tasas de formación de blastocistos (rango: 29’3 ± 3’0% - 36’9 ± 2’9%) o número total de células por blastocisto (rango: 44’6 ± 3’3- 47’6 ± 3’3).

Experimento 2. Evaluación de la viabilidad de ovocitos porcinos después de la vitrificación en EG+DMSO o EG+PG y el subsecuente desarrollo embrionario.

Diseño experimental

El segundo experimento fue diseñado para evaluar la viabilidad y los parámetros de fecundación y desarrollo embrionario de ovocitos (n=1198) vitrificados en estadio de VG usando las dos combinaciones de CPAs (EG+DMSO, EG+PG).

Para ello, se realizaron 4 replicados. Los ovocitos fueron equilibrados y vitrificados en los medios de vitrificación descritos en material y métodos. Los ovocitos del grupo control no fueron vitrificados. Tanto el grupo control como los ovocitos vitrificados fueron seleccionados y evaluados como en el experimento 1. Algunos de los ovocitos fueron fijados a las 18 horas para evaluar los parámetros de fecundación. El resto de presuntos cigotos fue cultivado para evaluar la tasa de división, la formación de blastocistos y el número total de células.

Resultados

La viabilidad 2 horas después del calentamiento fue menor (P < 0’001) para los ovocitos de los grupos vitrificados (67’0 ± 2’3% y 57’6 ± 2’3%; EG+DMSO y EG+PG, respectivamente) que para los ovocitos del grupo control (97’3 ± 0’7%). Además, la viabilidad FDA fue mayor (P < 0’01) cuando los ovocitos fueron vitrificados usando EG + DMSO en vez de EG + PG. En ambos grupos experimentales, EG + DMSO y EG + PG, los porcentajes de ovocitos vivos degenerados a las 44 horas de maduración (59’8 ± 2’3% and 56’2 ± 2’6%, respectivamente) fueron similares. Sin embargo, estos porcentajes fueron más altos (P < 0’001) que los observados en el grupo control (1’6 ± 1’3%). Estos ovocitos degenerados fueron eliminados antes de la FIV. A las 18 horas después de la FIV, un mayor número de ovocitos del grupo EG + DMSO (53’3 ± 5’0%) fue considerado degenerado comparado con los ovocitos del

grupo EG + PG y del grupo control (9’2 ± 0’3% and 2’4 ±0’9%, respectivamente). El porcentaje de ovocitos inmaduros fue mayor (P < 0’05) en el grupo EG + DMSO (25’0 ± 5’8%) que en el control (8’8 ± 1’7%) y en el grupo EG + PG (17’7 ± 3’6%). No hubo diferencias entre grupos en la penetración (rango: 57’0 ± 11’3% - 73’3 ± 2’8%) y monospermia (rango: 63’3 ± 2’3% - 67’9 ± 5’0%). Sin embargo, los ovocitos vitrificados usando EG + DMSO mostraron menores (P < 0’01) tasas de eficiencia (26’3 ± 7’7%) después de la FIV, comparadas con el grupo control y el grupo EG + PG (42’0 ± 2’2% and 39’6 ± 2’4%, respectivamente). El número total de células por blastocisto (rango: 32’8 ± 4’4 - 53’1 ± 2’6) no fue alterado por la vitrificación. Sin embargo la eficiencia fue mayor en el grupo control (31’9 ± 1’7%) que en el grupo EG + DMSO (6’6 ± 2’5%) y el grupo EG + PG (4’7 ± 1’6%). Además, la tasa de divididos y la formación de blastocistos fueron mayores (P < 0’001) en el grupo control (53’4 ± 2’7% y 61’8 ± 5’3%, respectivamente) que en los ovocitos vitrificados (grupo EG + DMSO: 25’9 ± 3’5% y 24’1 ± 10’0%; grupo EG + PG: 20’2 ± 5’8% y 27’2 ± 8’5% EG + PG).

OBJECTIVO 2. Evaluar el efecto de tres IRMs, dbcAMP, cicloheximida y cilostamida, y sus interacciones con las Gns sobre la maduración meiótica y el desarrollo embrionario de ovocitos porcinos. (Artículo 2).

Experimento 1. Evaluación de los efectos de la interacción de los IRMs y las Gns sobre la maduración nuclear de ovocitos.

Diseño experimental

Este experimento fue diseñado para evaluar los efectos de tres inhibidores reversibles de la meiosis y su interacción con las Gns sobre la maduración meiótica. Para ello, un total de 2058 ovocitos fueron madurados durante 22 horas en ausencia (control) o presencia de dbcAMP (1mM), cilostamida (20 µM) o cicloheximida (7µM) con o sin Gns, y durante otras 22 horas sin IRMs y Gns. La progresión nuclear se evaluó a las 22 horas y a las 44 horas de la maduración.

Resultados

La presencia de IRMs afectó (P < 0’005) al porcentaje de ovocitos en VG y estadios intermedios a las 22 horas de la maduración. Por lo contrario, la suplementación con Gns y la interacción IRMs-Gn no tuvo efectos significativos en estos parámetros. Sin la presencia de IRMs, las Gns produjeron una disminución (P < 0’02) en el porcentaje de ovocitos que

permanecieron en el estadio de VG. Sin embargo, la mayoría de ovocitos cultivados con IRMs permanecieron en estadio de VG (rango: 93’1 ± 5’7% - 99’4 ± 1’2%), independientemente de la presencia de Gns en el medio. A las 44 horas de la maduración, el porcentaje de ovocitos maduros (MII) fue mayor (P < 0’03) cuando se usaron Gns independientemente de la presencia o ausencia de IRMs. Entre los distintos inhibidores, la presencia de dbAMP consiguió una mayor (P < 0’05) tasa de ovocitos en MII comparado con la cilostamida (92’3 ± 3’1% y 63’1 ± 19’9%, respectivamente). En ausencia de Gns, la adición de cilostamida provocó un aumento (P < 0’05) de ovocitos en VG comparado con el control (80’3 ± 8’3% y 41’9 ± 11’5%, respectivamente) a diferencia de la adición de cicloheximida que provocó una disminución de ovocitos en VG (12’5 ± 2’9%). Sin embargo, en presencia de Gns, no hubo diferencias en las tasas de maduración de los distintos inhibidores con el grupo control.

Experimento 2. Evaluación de los efectos de la interacción de los IRMs con las Gns sobre el desarrollo embrionario de ovocitos porcinos.

Diseño experimental

El segundo experimento fue diseñado para evaluar los efectos de los IRMs y las Gns sobre el desarrollo embrionario de los ovocitos. En este experimento, un total de 2515 ovocitos madurados, como se explicó en el experimento 1, fueron fecundados y cultivados para evaluar su desarrollo embrionario. En cada replicado, parte de los cigotos fueron fijados y teñidos a las 18 horas después de la fecundación (n=1155) para evaluar los parámetros de fecundación. El resto de cigotos (n= 1360) se cultivaron para evaluar el desarrollo *in vitro* a los 2 y 7 días de cultivo.

Resultados

La presencia de IRMs, Gns y la interacción entre ambos factores (P < 0’01) afectó a las tasas de penetración y a la eficiencia de la fecundación. En presencia de Gns, solo la adición de dbcAMP produjo un mayor porcentaje (P < 0’05) de ovocitos penetrados (95’5 ± 6’5%) y eficiencia de la fecundación (49’4 ± 6’4%) comparado con los ovocitos madurados con Gns y sin IRMs (72’6 ± 7’1% and 33’7 ± 5’0%, respectivamente).

En ausencia de Gns, la adición de dbcAMP y de cicloheximida proporcionó un mayor porcentaje (P < 0’05) de ovocitos penetrados (80’9 ± 7’8% y 81’7 ± 14’4%) respecto al control (56’5 ± 7’3%), pero no hubo diferencias en la eficiencia total del sistema entre estos grupos

(23’4 ± 2’8%, 30’0 ± 3’7% and 19’6 ± 2’9%, respectivamente). Por el contrario, la cilostamida, en ausencia de Gns disminuyó (P < 0’05) el porcentaje de peneración (27’2 ± 11’1%) y la eficiencia (12’0 ± 6’4%) con respecto al control (56’5 ± 7’3% y 19’6 ± 2’9%, respectivamente).

La presencia de IRMs, Gns y la interacción entre ambos factores afectó (P < 0’05) todos los parámetros de desarrollo embrionario evaluados. La presencia de dbcAMP durante la maduración incrementó (P < 0’05) la formación de blastocistos y la eficiencia de formación de blastocistos en presencia (59’9 ± 7’4% y 45’1 ± 7’7%, respectivamente) y ausencia (40’8 ± 15’0% y 22’3 ± 8’6%, respectivamente) de Gns, comparado con los controles (38’9 ± 11’4% y 26’3 ± 8’6% en presencia de Gns y 18’8 ± 3’1% y 6’2 ± 2’3% en ausencia de Gns). Además los ovocitos madurados con dbcAMP y Gns tuvieron una mayor (P < 0’001) eficiencia de formación de blastocistos (45’1 ± 7’7%) comparado con los otros grupos tratados (15’6 ± 10’9% y 27’4 ± 6’8%, cicloheximida y cilostamida, respectivamente). No se observaron diferencias en el número total de células entre grupos (rango: 31’7 ± 2’8 - 46’3 ± 5’8).

OBJETIVO 3. Determinar los efectos de la adición de AsA, en los medios de maduración, fecundación y cultivo embrionario sobre los parámetros de maduración, fecundación y desarrollo embrionario, así como el efecto de la adición de dicho antioxidante en los medios de vitrificación sobre la viabilidad después del calentamiento (Artículo 3).

Experimento 1. Efectos de la adición de AsA en MIV, FIV y CIV sobre la maduración, fecundación y desarrollo embrionario.

Diseño experimental

En el primer experimento, se evaluaron los efectos de la suplementación del AsA en los medios de MIV, FIV y CIV. Para ello, se llevó a cabo la maduración, fecundación y cultivo de los ovocitos en presencia o ausencia de 50 µg/mL de AsA en todas las posibles combinaciones, lo que supuso un total de 8 grupos experimentales. Se utilizaron un total de 2744 ovocitos en seis replicados. Un número aleatorio de ovocitos (n=149) y de presuntos cigotos (n=1142) de cada grupo se fijó y tiñó a las 44 horas de la MIV y a las 18 horas después de la FIV para evaluar maduración y parámetros de fecundación, respectivamente. El resto de presuntos cigotos (n=1602) fueron cultivados para evaluar el desarrollo embrionario. El día 7 los blastocistos fueron fijados y teñidos para evaluar el número total de células.

Resultados

La adición de AsA no tuvo efecto en el porcentaje de ovocitos en MII a las 44 horas de maduración entre el grupo tratado (81’4%) y el control (83’5%). La suplementación de los medios de MIV, FIV y CIV tampoco afectó a los parámetros de fecundación. La tasa de penetración estuvo cerca del 70% y la monospermia alrededor del 60% en todos los grupos. La eficiencia total de la FIV osciló entre el 37’6 ± 8’5% y el 47’3 ± 13’9%, sin diferencias entre los grupos experimentales y el control. La adición de AsA tampoco afectó el desarrollo embrionario hasta el estadio de 2-4 células, ni a la formación de blastocistos al final del periodo de cultivo. La eficiencia total de blastocistos estuvo alrededor del 30% en todos los casos. La calidad de los blastocistos producidos *in vitro* en términos de número de células (rango: desde 44’1 ± 20’4 hasta 53’0 ± 26’2 células) tampoco sufrió variación por la suplementación de AsA.

Experimento 2. Efectos de la adición de AsA en los medios de vitrificación y calentamiento sobre la supervivencia y calidad de los blastocistos producidos *in vitro*.

Diseño experimental

Para ello, se llevó a cabo una PIV de embriones sin la presencia de AsA. El día 6, los blastocistos (n=588) procedentes de seis replicados distintos se dividieron aleatoriamente en dos grupos, en uno se suplementaron los medios de vitrificación y calentamiento con 50 µg/mL de AsA (grupo VW+) y en el otro no se suplementaron (grupo control, VW-). Después del calentamiento, los blastocistos de los grupos VW+ (n=281) y VW- control (n=307) fueron cultivados *in vitro* durante 24 horas para evaluar la viabilidad y las tasas de división. Un número aleatorio de blastocistos vitrificados y calentados (n=21) y clasificados como viables de cada grupo fueron sometidos a tinción diferencial para evaluar el número total de células, el número de células de la MCI y el número de células del trofoectodermo. Finalmente, se evaluaron los niveles de ROS y GSH en ambos grupos y de algunos blastocistos producidos *in vitro* (control) no vitrificados.

Resultados

La adición de AsA durante la vitrificación y el calentamiento aumentó (P<0'05) la viabilidad de los blastocistos producidos *in vitro* después del calentamiento comparado con el grupo VW-control (51'1 ± 20'9% and 34'8 ± 21'4%, respectivamente). El número total de células (58'7 ± 21'1 y 62'6 ± 14'4, VW+ y VW-control, respectivamente) y la distribución de células entre del trofoectodermo y la MCI fueron similares entre ambos grupos. La vitrificación y el calentamiento incrementaron (P < 0'05) los niveles de ROS y disminuyeron (P < 0'05) los niveles de GSH, comparados con los controles. La adición de AsA a los medios de vitrificación y calentamiento disminuyó (P < 0'05) la producción de ROS, pero no afectó a la producción de GSH. Aquellos embriones vitrificados y calentados sin AsA (VW- control) presentaron mayores (P < 0'05) niveles de ROS intracelular que aquellos tratados con AsA, que a su vez presentaron mayores niveles de ROS que los ovocitos no vitrificados.

OBETIVO 4. Investigar los efectos del número de partos, la estación del año y el intervalo destete-celo sobre los parámetros embrionarios en día 6 después de la inseminación de donantes superovuladas en el celo post-destete (Artículo 4).

Diseño experimental

Este experimento fue llevado a cabo durante un periodo de 3 años y en 18 replicados usando un total de 221 cerdas donantes, con un periodo de lactación de 21'8 ± 0'1 días. En cada replicado, solo se seleccionaron las cerdas con un intervalo destete-celo de 3 ó 4 días. Las donantes fueron seleccionadas en el intervalo del 20 de Marzo al 20 de Junio (primavera), del 23 de Septiembre al 20 de Diciembre (otoño) y del 21 de Diciembre al 19 de Marzo (invierno) en dos replicados por estación y por año. La selección de donantes se basó en su historial reproductivo (promedio de fertilidad y tamaño de las camadas > 90% y 10 lechones, respectivamente), la condición corporal (entre 2'75 y 3'25 sobre una escala de 5, en el día del destete) y número de partos (de 2 a 7 partos).

Resultados

De todas la donantes superovuladas (n=221), el 82'8% (n=183) mostró signos de estro en los días 3 ó 4 después del destete (42'1% y 40'7%, respectivamente). El resto de cerdas (17'2%) mostraron signos de estro después del día 4 post-destete y no fueron utilizadas para

este estudio. De 183 cerdas donantes, 179 (97’8%) tenían embriones en día 6 después de la inseminación, y solo 4 (2’2%) tuvo ovocitos después de los lavados. La proporción de donantes con quistes ováricos fue del 23’0 % con una media de 3’5 ± 0’2 quistes por cerda. La tasa de ovulación fue del 23’8 ± 0’4 cuerpos lúteos (rango de 6 a 40 cuerpos luteos, CV=24.0%). La tasa de recolección y la tasa de fecundación fue de 90’8 ± 0’8% y 96’3 ± 0’9%, respectivamente, y la media de embriones viables y ovocitos o embriones degenerados obtenidos en las cerdas gestantes fue 20’8 ± 0’4 y 1’0 ± 0’1, respectivamente. La proporción de embriones transferibles en relación al número de embriones viables fue de 94’3 ± 1’4%. El número total de embriones transferibles recolectados de las cerdas donantes inseminadas (N=183) fue de 3508. No hubo diferencias significativas en el número de partos, estación, intervalo destete-celo o sus interacciones sobre la gestación, numero de quistes en cerdas con quistes o los parámetros embrionarios estudiados.

Respecto a las estimaciones de riesgo de la estación, número de partos e intervalo destete-celo en relación al desarrollo de quistes ováricos en las cerdas donantes, el análisis de regresión logística multivariable mostró que el número de partos y el intervalo destete-celo no estuvieron asociados con las tasas de quistes ováricos. Por el contrario, la estación del año estuvo significativamente asociada con los quistes ováricos con un aumente del 3’5 a un 5’5 de riesgo de tener quistes ováricos en otoño (ORs=3’69, 95% ICs, 1’46 a 9’37) e invierno (ORs=5’57, 95% ICs, 2’00 a 15’51), respectivamente, comparado con primavera.

OBJETIVO 5. Explorar la posibilidad de re-vitrificar embriones porcinos obtenidos *in vivo* (morulas y blastocistos no eclosionados). (Artículo 5).

Diseño experimental

Los embriones recolectados (n=255) se clasificaron en dos grupos: un grupo que se sometió a una sola vitrificación y calentamiento (VW, grupo de control de vitrificación) y un grupo control de embriones frescos. Después, cada grupo se dividió en dos subgrupos de acuerdo con su etapa de desarrollo embrionario (mórula o blastocito). Los embriones del grupo de control fresco no se vitrificaron, y se evaluó la viabilidad embrionaria a las 24 horas de CIV. Del grupo VW se evaluaron las tasas de viabilidad y eclosión de los embriones a las 24 horas después del calentamiento. Posteriormente, los blastocistos viables no eclosionados del grupo VW se volvieron a vitrificar y recalentar (grupo experimental RVW; n=136) y se evaluó la

viabilidad de esos embriones a las 24 horas de CIV. Finalmente, evaluamos las tasas de eclosión de los embriones de los grupos RVW y control fresco a las 48 horas de CIV.

Resultados

Una alta proporción (> 85%) de embriones sobrevivió a la primera vitrificación y calentamiento y logró tasas de supervivencia comparables a las de sus controles frescos. Por el contrario, una posterior segunda vitrificación y calentamiento fue perjudicial para los embriones, ya que sus tasas de supervivencia (63’9% para mórulas y 60’0% para blastocistos) se redujeron significativamente ($P < 0'01$), en comparación con los de los grupos de control. A las 48 horas de cultivo, las tasas de eclosión fueron similares en blastocistos re-vitrificados derivados de mórulas vitrificadas y del grupo control fresco (50’8% y 55’3%, respectivamente). Se registraron diferencias significativas ($P < 0'01$) en las tasas de eclosión en blastocistos re-vitrificados derivados de blastocistos vitrificados y blastocistos control (14’7% y 90’0%, respectivamente).

OBJETIVO 6. Evaluar la eficacia de los tanques de nitrógeno seco para mantener la viabilidad y la calidad de embriones porcinos obtenidos *in vivo* (morulas y blastocistos no eclosionados) y vitrificados durante un periodo de 3 días. (Artículo 5).

Experimento 2. Evaluación de la eficacia de un tanque seco de nitrógeno para mantener la viabilidad de embriones vitrificados durante 3 días.

Diseño experimental

Después de la vitrificación, las pajuelas SOPS que contenían los embriones se almacenaron en un tanque de NL_2 durante un mes. Posteriormente, las pajuelas se trasladaron desde el tanque de NL_2 al DS (grupo DS, n=102) o a otro tanque NL_2 (grupo control, n=101) durante tres días adicionales. En ambos grupos, los embriones se dividieron según su etapa de desarrollo (mórula o blastocito). Después del calentamiento, los embriones del DS y los del grupo control se cultivaron durante 24 horas para evaluar la supervivencia del embrión y las tasas de eclosión. Finalmente, una alícuota de embriones de cada grupo se sometió a un ensayo TUNEL para evaluar los núcleos con degradación del ADN y el índice apoptótico.

Resultados

Los embriones del grupo DS presentaron tasas de supervivencia similares a las del grupo control NL_2 (rango: 88-100%), independientemente de la etapa embrionaria analizada. El almacenamiento de mórulas y blastocistos en DS no afectó al desarrollo embrionario al final del cultivo en comparación con el grupo control. El patrón de la distribución de frecuencia del desarrollo embrionario fue comparable entre los grupos. Aunque no hubo diferencias en el número total de células de los blastocistos resultantes entre los grupos DS y NL_2 (rango: 119’9 ± 60’6 - 163’1 ± 39’2), el índice apoptótico se vio afectado, siendo ligeramente mayor ($P < 0’05$) en las morulas y los blastocistos almacenados en el DS (3’0 ± 2’6 y 2’6 ± 2’6, respectivamente) comparados con el grupo NL_2 (1’3 ± 1’8 y 1’7 ± 1’9, respectivamente) independientemente de la etapa de desarrollo.

OBJETIVO 7. Investigar en cerdas receptoras cómo influye el número de partos en su capacidad reproductiva después de la NsDU-TE (Articulo 6).

Diseño experimental

Tras el destete, las cerdas receptoras (n=120) fueron seleccionadas según su historial reproductivo (fertilidad previa > 90% y tamaño de la camada previo de > 9 lechones) y según su condición corporal (condición corporal de 2’75 a 3’25 sobre una escala de 5 puntos el día del destete) y se agruparon en tres categorías de acuerdo al número de partos: primíparas, cerdas de dos partos y cerdas de 3 a 5 partos. Treinta embriones transferibles (mórulas y blatocistos no eclosionados) fueron transferidos de manera no quirúrgica en un cuerno uterino en cada receptora.

El mismo operador llevó a cabo todas las transferencias. El experimento supuso un total de 7 replicados. En cada replicado, las donantes fueron inseminadas con dosis espermáticas del mismo verraco, los embriones procedentes de cada donante fueron divididos aleatoriamente en los grupos receptores. Un número similar de receptoras fue seleccionado para cada uno de los tres grupos. Las dificultados durante la inserción de la sonda de transferencia no quirúrgica a través del cérvix y el cuerno uterino fue evaluada de acuerdo a la sensibilidad frente a la resistencia que consideró el operador mientras manipulaba la sonda y se dividió en tres clases (1: nula o baja resistencia, 2: moderada o severa resistencia, 3:

imposible la inserción a través del cérvix). La posición de la sonda de transferencia no quirúrgica en el cuerno uterino fue considerada correcta cuando no se observaron bucles en la sonda después de extraerla del útero (Martinez et al., 2004). La presencia de sangre en la sonda durante o después de las inserciones y la incidencia de descargas vaginales después de las transferencias fueron también evaluadas.

A los 12 días después de la transferencia no quirúrgica, las receptoras fueron evaluadas diariamente para observar signos de estro. Las cerdas gestantes fueron diagnosticadas mediante ultrasonografía los días 20-22 después de la transferencia. Se permitió que Todas las cerdas gestantes se permitieron llegar a término y los datos referentes a tasas de retorno al estro (regular e irregular), tasas de abortos, tasas de partos y tamaño de las camadas fueron recopilados. La eficiencia de la producción de lechones fue calculada como el ratio del número total de lechones nacidos vivos respecto al número de embriones transferidos en todas las receptoras.

Resultados

De las 158 donantes, se recogieron embriones de 148 (93'7%) el día 6 después de la inseminación, 9 (5'7%) tenían solo ovocitos después del lavado y 1 (0'6%) tenía ovarios poliquísticos sin cuerpos lúteos en sus ovarios. La tasa media de ovulación fue de 25'7 ± 5'0 cuerpos lúteos (rango: 15 a 61 cuerpos lúteos, CV=19'4%). La tasa de recuperación fue de 97'0%, y el promedio de embriones viables, y ovocitos y / o embriones degenerados obtenidos en las cerdas gestantes fue de 23'0 ± 3'1 y 1'8 ± 1'8, respectivamente (92'8% tasa de fertilización). La proporción de embriones transferibles (mórulas compactadas y blastocistos no eclosionados) en relación con el número de embriones viables fue del 98'4%.

Los embriones viables no transferibles se relacionaron exclusivamente con la presencia de mórulas no compactadas y blastocistos eclosionados. El número total de embriones transferibles de los donantes inseminados (n=158) fue de 3364, lo que resultó en una relación de donante a receptor de 1'4: 1. Las dificultades encontradas durante la inserción del catéter NsDU-ET se consideraron nulas o menores en el 90'0% de las transferencias. El número de partos previos [1 (n=41), 2 (n= 43) y 3 a 5 (n=36)] no afectó el grado de dificultad de las inserciones, siendo más del 80% de la clase 1, menos del 15% de clase 2 y menos del 5% de clase 3. Dos de 120 (1'7%) receptoras fueron excluidas del estudio porque no fue posible insertar el catéter a través del canal cervical. El catéter se insertó correctamente en uno de los

cuernos uterinos en 111 de las 118 (94’1%) receptoras restantes, sin diferencias entre los distintos grupos. Además, no se sospecharon perforaciones del cuello uterino o la pared uterina durante las inserciones del catéter, el comportamiento de las hembras receptoras durante el procedimiento se clasificó como bueno (sin reacción o reacción menor) en todos los casos, y no se observaron descargas vaginales después del procedimiento en ninguna de las cerdas receptoras. Con respecto a los resultados reproductivos de las receptoras con una inserción adecuada, el número de receptoras evaluadas fue: 38 cerdas de un parto, 40 cerdas de dos partos y 33 cerdas de 3 a 5 partos. No hubo diferencias en las tasas de gestación (rango: 73’7% - 75’8%), tasas de partos (rango: 68’4% - 72’7%), o tamaños de camadas (rango: 9’5 ± 3’3 - 9’8 ± 3’4 lechones nacidos) entre grupos. Además, no hubo diferencias entre los grupos con respecto al peso al nacer de los lechones (rango: 1’5 ± 0’3 kg - 1’7 ± 0’3 kg) o la eficiencia de la producción de lechones (rango: 22’3% - 23’0%).

CONCLUSIONES

1. En ausencia de vitrificación, los efectos tóxicos de los medios de vitrificación sobre ovocitos en estadio de VG fueron mínimos y similares para ambas combinaciones de CPA. Los ovocitos clasificados como viables después de la exposición a los CPA y después de la MIV mostraron tasas similares de fecundación y desarrollo embrionario a los controles.

2. Se pueden obtener blastocistos de alta calidad mediante la vitrificación de ovocitos inmaduros por el sistema SOPS. Sin embargo, la tasa de blastocistos fue muy baja y el desarrollo embrionario de los ovocitos vitrificados fue menor comparado con el de los controles.

3. La interacción de las Gns con los tres inhibidores reversibles de la meiosis (dbcAMP, cicloheximida y cilostamida) aceleraron la progresión meiótica hasta el estadio de MII. La presencia de dbcAMP durante la primera fase de la maduración puede incrementar, incluso duplicar la capacidad de los ovocitos para alcanzar el estadio de blastocisto.

4. Bajo nuestras condiciones de laboratorio, la suplementación de medio de IVM/IVF/IVC con AsA en una concentración de 50μg/mL no mejoró los resultados de la PIV de embriones. Por el contrario, la adición de AsA a los medios de vitrificación y calentamiento aumentó la supervivencia después de la vitrificación mediante la disminución de la producción de ROS.

5. El número de partos de la donante no afectó las tasas de gestación y de fecundación así como el número y calidad de los embriones de día 6, independientemente de la estación del año (desde otoño a primavera) o del IDC (3 o 4 días).

6. Los blastocistos porcinos derivados de morulas y los blastocistos vitrificados y calentados pueden ser re-vitrificados con buenas tasas de supervivencia.

7. El DS es un eficiente sistema para almacenar morulas y blastocistos vitrificados durante un periodo de tres días, sin afectar la viabilidad después del calentamiento.

8. Las tasas de gestación y fecundación así como el tamaño de camada no se vieron afectadas por el número de partos de las receptoras después de la transferencia no quirúrgica de embriones.

ABREVIATURAS

AsA: Ácido ascórbico

BSA: Albúmina sérica bovina

Br-dUTP: Bromodioxiuridina trifosfato

CIV: Cultivo in vitro

CCOs: Complejo células-ovocitos

CPAs: Crioprotectores

DbcAMP: Monofosfato de adenosina cíclico dibutirílico

DE: Desviación estándar

DMSO: Dimetilsulfóxido

DSs: Dry shippers

EG: Etilenglicol

FDA: Diacetato de fluoresceína

FIV: Fecundación *in vitro*

Gns: Gonadotropinas

ICs: Intervalos de confianza

IDC: Intervalo destete celo

IRMs: Inhibidores reversibles de la meiosis

mTBM: Modified Tris-buffered medium

MII: Metafase II

MCI: Masa celular interna

MIV: Maduración *in vitro*

NCSU23,37: North Caroline State University medium

NL_2: Nitrógeno líquido

NsDU: Transferencia no quirúrgica intrauterina profunda

OR: Odds ratios

PBSD: Dulbecco´s phosphate-buffered saline medium

PG: Propilenglicol

PIV: Producción *in vitro*

ROS: Reactive oxygen species

SEM: Error estándar de la media

SOPS, OPS: Super Open pull straw, Open pull straw

TE: Transferencia de embriones

TL-HEPES-PVA: Tyrode´s lactate-HEPES PVA

V1: Primer medio de vitrificación

V2: Segundo medio de vitrificación

VG: Vesícula Germinal

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ARTICLES

ARTÍCULO 1

Theriogenology

Effects of two combinations of cryoprotectants on the *in vitro* developmental capacity of vitrified immature porcine oocytes

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THERIOGENOLOGY

ANIMAL REPRODUCTION

ARTÍCULO 2

ORIGINAL ARTICLE

Effects of meiotic inhibitors and gonadotrophins on porcine oocytes in vitro maturation, fertilization and development

MA Gil | A Nohalez | CA Martinez | JR Ake-Villanueva | F Centurion-Castro | C Maside | C Cuello | J Roca | I Parrilla | EA Martinez

Reproduction in Domestic Animals

ARTÍCULO 3

Theriogenology

Exogenous ascorbic acid enhances vitrification survival of porcine in vitro-developed blastocysts but fails to improve the in vitro embryo production outcomes

THERIOGENOLOGY

ANIMAL REPRODUCTION

ARTÍCULO 4

Factors of importance when selecting sows as embryo donors

animal

ARTÍCULO 5

Theriogenology

Eventual re-vitrification or storage in liquid nitrogen vapor does not jeopardize the practical handling and transport of vitrified pig embryos

THERIOGENOLOGY

ANIMAL REPRODUCTION

ARTÍCULO 6

Reproduction in Domestic Animals

The Recipients' Parity Does Not Influence Their Reproductive Performance Following Non-Surgical Deep Uterine Porcine Embryo Transfer

Reproduction in Domestic Animals

ARTICLE 1

Effects of two combinations of cryoprotectants on the *in vitro* developmental capacity of vitrified immature porcine oocytes.

Journal Theriogenology. 2015; 84:545-52.

Abstract

This study evaluated two cryoprotectant (CPA) combinations, ethylene glycol (EG) + DMSO and EG + propylene glycol (PG), used for the vitrification of germinal vesicle (GV) porcine oocytes. In experiment 1, the equilibration of GV with the two CPA combinations increased ($P < 0.05$) the percentage of oocytes that degenerated after IVM (18.1 ± 2.3% and 19.4 ± 2.6% for EG + DMSO and EG + PG groups, respectively) compared with control oocytes (7.6 ± 1.3%). However, CPAs did not affect the fertilization or developmental parameters of the embryos. In experiment 2, the percentages of live vitrified-warmed GV oocytes at 2 hours after warming (EG + DMSO: 67.0 ± 2.3% and EG + PG: 57.6 ± 2.3%) were lower than those of fresh control GV oocytes (97.3 ± 0.7%). The percentage of degenerated oocytes after IVM was higher ($P < 0.001$) in vitrified-warmed oocytes (EG + DMSO: 59.8 ± 2.3% and EG + PG: 56.2 ± 2.6%) than in the control (1.6 ± 1.3). Fertilization efficiency was higher ($P < 0.05$) in the EG + PG (39.6 ± 2.4%) and control (42.0 ± 2.2%) groups than in the EG + DMSO (26.3 ± 7.7%) group. The cleavage and blastocyst formation rates of the EG + DMSO (25.9 ± 3.5% and 6.6 ± 2.5%, respectively) and EG + PG (20.2 ± 5.4% and 4.7 ± 1.6%, respectively) vitrification groups were lower ($P < 0.001$) than those observed in the control oocytes (53.4 ± 2.7% and 31.9 ± 1.7%, respectively). In conclusion, in the absence of vitrification, the toxic effects of both CPA combinations on the GV oocytes were minimal. Vitrification resulted in important losses in viability at each step of the in vitro embryo production procedure. However, the surviving oocytes were able to mature and be fertilized, although the fertilization efficiency in the EG + DMSO group was lower. Blastocysts formation was similar for both CPA combinations.

URL https://www.ncbi.nlm.nih.gov/pubmed/25998270

ARTICLE 2

Effects of meiotic inhibitors and gonadotrophins on porcine oocytes in vitro maturation, fertilization and development.

Journal Reproduction in Domestic Animals**.** 2017; 52:873-880.

Abstract

This study evaluated the effect of three reversible meiotic inhibitors (MINs) and their interaction with gonadotrophins (Gns) on the meiotic maturation and developmental competence of porcine oocytes. In experiment 1, the oocytes were matured for 22 hr in the presence or absence of dbcAMP (1 mM), cycloheximide (7 µM) or cilostamide (20 µM) with or without Gns, and for an additional 22 hr in the absence of MINs and Gns. At 22 hr of maturation, regardless of the presence of Gns, a higher proportion ($p < .001$) of oocytes cultured in the presence of MINs were effectively arrested at the germinal vesicle stage compared with the oocytes cultured without MINs. At 44 hr of maturation, the proportion of oocytes that reached MII was higher ($p < .05$) in groups with Gns compared with groups without Gns. In experiment 2, oocytes that were matured as in experiment 1 were inseminated and cultured for 7 days to evaluate fertilization parameters and blastocyst formation. Only oocytes from the dbcAMP + Gns group had higher ($p < .05$) efficiency of fertilization compared with the other treatment groups. The presence of dbcAMP during maturation also increased ($p < .05$) blastocyst formation and efficiency of blastocyst formation in both the presence and absence of Gns. These results indicate that the interaction of Gns with the tested MINs improved meiotic progression. In addition, regardless of supplementation with Gns, the presence of dbcAMP during the first maturation period increased and even doubled the capacity of oocytes to develop to the blastocyst stage.

URL https://www.ncbi.nlm.nih.gov/pubmed/28543962

ARTICLE 3

Exogenous ascorbic acid enhances vitrification survival of porcine in vitro-developed blastocysts but fails to improve the in vitro embryo production outcomes.

Journal Theriogenology. 2018; 6:113-119

Abstract
In this study, the effects of addition of the antioxidant ascorbic acid (AsA) were evaluated during porcine in vitro embryo production (IVP) and vitrification. In experiment 1, the effects of AsA supplementation during IVM, IVF and IVC were evaluated, using a total of 2744 oocytes in six replicates. The IVM, IVF and embryo IVC media were supplemented or not (control) with 50 µg/mL AsA in all possible combinations. No significant effects of AsA were detected in any of the maturation, fertilization or embryo development parameters assessed. In experiment 2, we evaluated the effects of adding AsA to vitrification-warming media on the post-warming survival and quality of blastocysts. Day-6 in vitro-produced blastocysts (N = 588) from six replicates were randomly divided in two groups, with vitrification and warming media either supplemented with 50 µg/mL AsA (VW + group) or un-supplemented (VW- control). Addition of AsA increased ($P < 0.05$) blastocyst survival rate after vitrification compared with that of VW- control embryos. Vitrification and warming increased ($P < 0.05$) the production of oxygen species (ROS) and reduced ($P < 0.05$) the glutathione levels in blastocysts. Although VW + blastocysts displayed higher ($P < 0.05$) ROS levels than those of fresh control blastocysts, the levels were lower ($P < 0.05$) than those found in VW- control blastocysts. In conclusion, under the experimental conditions, supplementation of IVM/IVF/IVC media with AsA did not improve the embryo production in vitro. By contrast, the addition of AsA to chemically defined vitrification and warming media increased the survival of in vitro-produced porcine blastocysts by decreasing ROS production.

URL https://www.ncbi.nlm.nih.gov/pubmed/29477909

ARTICLE 4

Factors of importance when selecting sows as embryo donors.

Journal Animal. 2017; 11:1330-1335.

Abstract

The improvement in porcine embryo preservation and non-surgical embryo transfer (ET) procedures achieved in recent years represents essential progress for the practical use of ET in the pig industry. This study aimed to evaluate the effects of parity, weaning-to-estrus interval (WEI) and season on reproductive and embryonic parameters at day 6 after insemination of donor sows superovulated after weaning. The selection of donor sows was based on their reproductive history, body condition and parity. The effects of parity at weaning (2 to 3, 4 to 5 or 6 to 7 litters), season (fall, winter and spring), and WEI (estrus within 3 to 4 days), and their interactions on the number of corpus luteum, cysts in sows with cysts, number and quality of viable and transferable embryos, embryo developmental stage and recovery and fertilization rates were evaluated using linear mixed effects models. The analyses showed a lack of significant effects of parity, season, WEI or their interactions on any of the reproductive and embryonic parameters examined. In conclusion, these results demonstrate that fertilization rates and numbers of viable and transferable embryos collected at day 6 of the cycle from superovulated donor sows are not affected by their parity, regardless of the time of the year (from fall to spring) and WEI (3 or 4 days).

URL https://www.ncbi.nlm.nih.gov/pubmed/28219466

ARTICLE 5

The Recipients' Parity Does Not Influence Their Reproductive Performance Following Non-Surgical Deep Uterine Porcine Embryo Transfer.

Journal Reproduction in Domestic Animals. 8;113:229-236

Abstract

With the development of the non-surgical deep uterine (NsDU) embryo transfer (ET) technology, the commercial applicability of ET in pigs is now possible. There are, nevertheless, many factors that influence NsDU-ET effectiveness that need to be addressed. The aim of this study was to evaluate the effects of the weaned recipients' parity on fertility and prolificacy following NsDU-ET. The recipients (n = 120) were selected based on their reproductive history and body condition and grouped into three categories according to their parity: primiparous sows, sows of parity 2 and sows of parities from 3 to 5. Thirty fresh embryos (morulae and unhatched blastocysts) were non-surgically transferred into one uterine horn of each recipient. It was possible to insert the NsDU-ET catheter through the cervix along a uterine horn in 98.3% of the recipients. The parity had no influence on the difficulty grade of the insertions or on the percentage of correct insertions. The cervix and uterine wall were not perforated during the insertions, and vaginal discharge was not observed after transfer in any of the recipients. There were no differences in the pregnancy rates (74.8%), farrowing rates (71.2%) or litter sizes (9.6 ± 3.3) between groups. Also, there were no differences between groups regarding to the piglets' birthweights or piglet production efficiency. In conclusion, these results demonstrate that weaned sows from parity 1 to 5 are appropriate to be used as recipients in NsDU-ET programs, which increase the possibilities for the utilization of ET in the recipient farms.

URL https://www.ncbi.nlm.nih.gov/pubmed/26661993

ARTICLE 6

Eventual re-vitrification or storage in liquid nitrogen vapor does not jeopardize the practical handling and transport of vitrified pig embryos.

Journal Theriogenology. 2016; 51:123-9.

Abstract

This study aimed (1) to evaluate the in vitro post-warming survival of porcine embryos after re-vitrification and (2) to assess the efficacy of transport of embryos in dry shipper (DS) in maintaining the viability and quality of vitrified embryos for a 3-day period. Embryos at the compacted or cavitating morula (CCM) and unhatched blastocyst (UBL) stages were surgically obtained from weaned, crossbred sows. In the first experiment, more than 85% of the embryos survived an initial vitrification and warming and achieved comparable survival rates to those of their fresh counterparts. In contrast, those embryos subjected to a second vitrification and warming had clearly lower survival rates (60% and 64% for re-vitrified embryos from the CCM and UBL groups, respectively) compared to the survival rates of the initial vitrification and fresh control groups ($P < 0.01$). Hatching rates were similar in re-vitrified blastocysts derived from vitrified CCMs and fresh control groups (50.8% and 55.3%, respectively). However, differences ($P < 0.01$) in hatching rates were recorded in re-vitrified blastocysts derived from vitrified UBLs and fresh control blastocysts (14.7% and 90.0%, respectively). In the second experiment, vitrified embryos were stored in a liquid nitrogen tank for one month. Then, the straws containing the embryos were transferred to a DS (DS group) or to another liquid nitrogen tank (control group) for an additional three days. Embryos from the DS and control groups had similar survival and hatching rates, regardless of the embryonic stage considered. The DS storage of CCMs and UBLs did not affect their development after culturing, including total cell numbers, compared to the control, although their apoptotic index was slightly higher ($P < 0.05$), regardless of the developmental stage. In conclusion, although re-vitrification negatively affects embryo survival, this study demonstrated that >60% of vitrified embryos could be successfully re-vitrified and re-warmed. The present study also showed the effectiveness of the DS for the storage of vitrified porcine CCMs and UBLs for at least three 3 days.

URL https://www.ncbi.nlm.nih.gov/pubmed/29567383

GRAPHIX APPENDIX

GRAPHIX APPENDIX

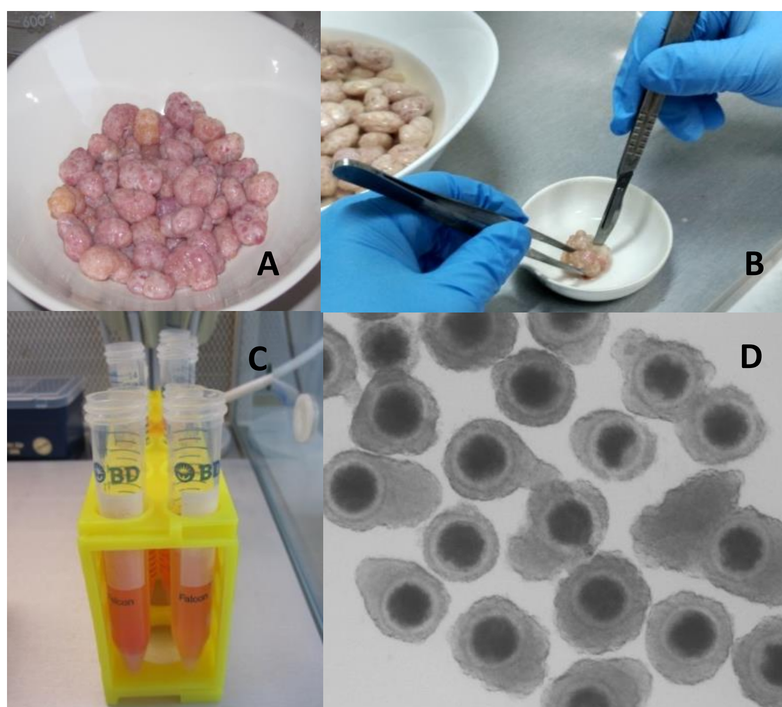

Figure 1

Collection of cumulus-oocytes complexes.

A, ovaries obtained from prepubertal gilts of a local slaughterhouse. B, collection of COCs using a sterile scalpel blade from the surfaces of medium-sized follicles. C, sedimentation of follicular fluid. D, selection of oocytes with a compact cumulus mass.

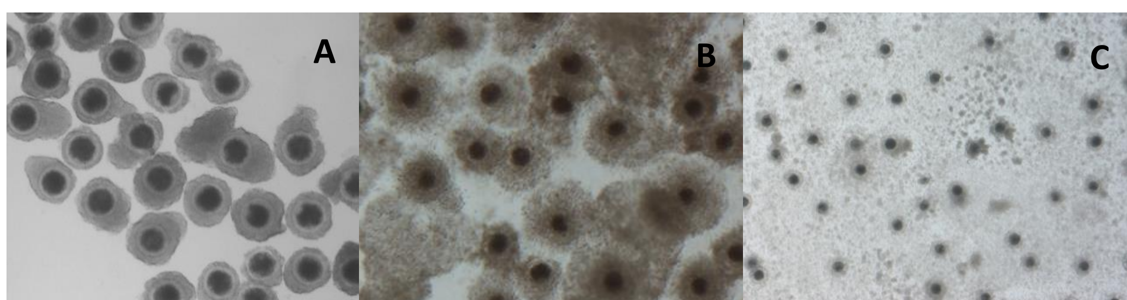

Figure 2.

In vitro maturation

A inmature cumulus-oocytes complexes; B, cumulus-oocytes complexes after 20-22 h of in vitro maturation; C, cumulus-oocytes complexes after 40-44 h of in vitro maturation.

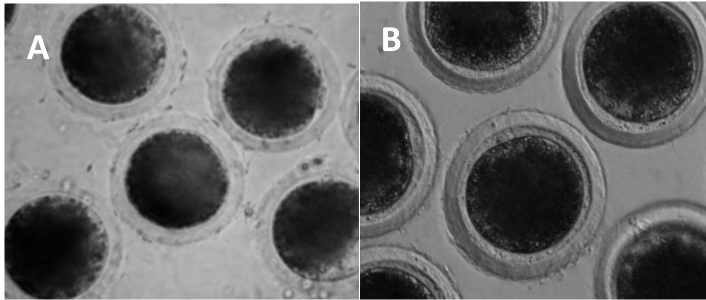

Figure 3

In vitro fertilization

A, Co-culture of denuded in vitro matured-oocyte and spermatozoa. B, presumptive zygote after 18 h of in vitro fertilization

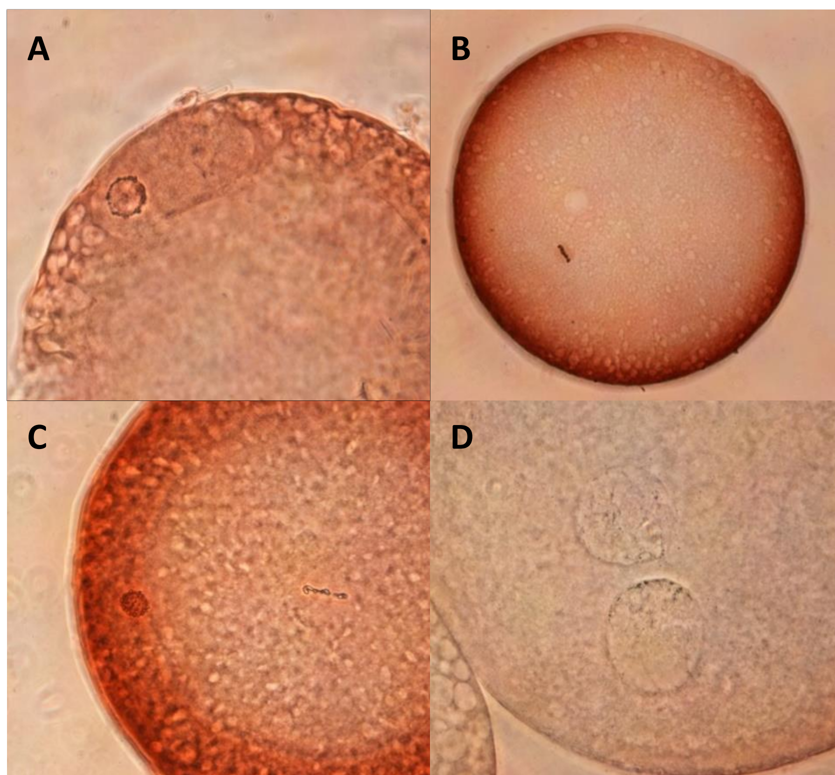

Figure 4

Maturation and Fertilization parameters

A, inmature oocyte at germinal vesicle. B, inmature oocyte with metaphase plate (arrow). C, in vitro matured oocytes with metaphase plate (arrow), and visible first polar body (asterisk). D, monospermic zygote with two pronuclei.

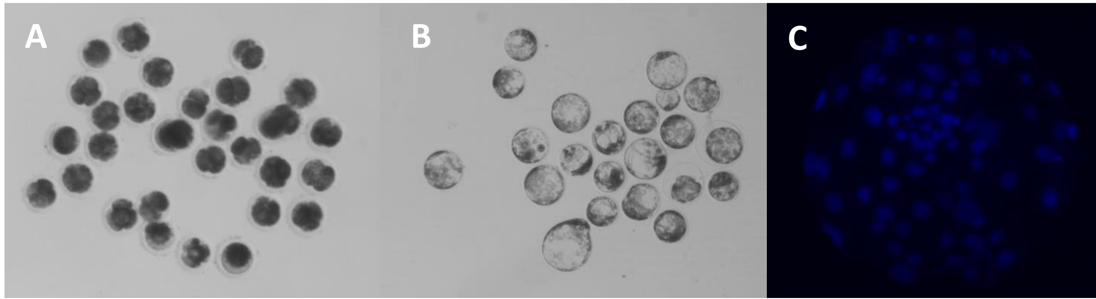

Figure 5

In vitro embryo development

A, Cleavage embryos form 2 to 4 cell at 48 h of in vitro culture; B, in vitro culture blastocysts; C, Assesment of total number of cells in in vitro blastocysts stained with Hoechst 33342 and ultraviolet irradiation.

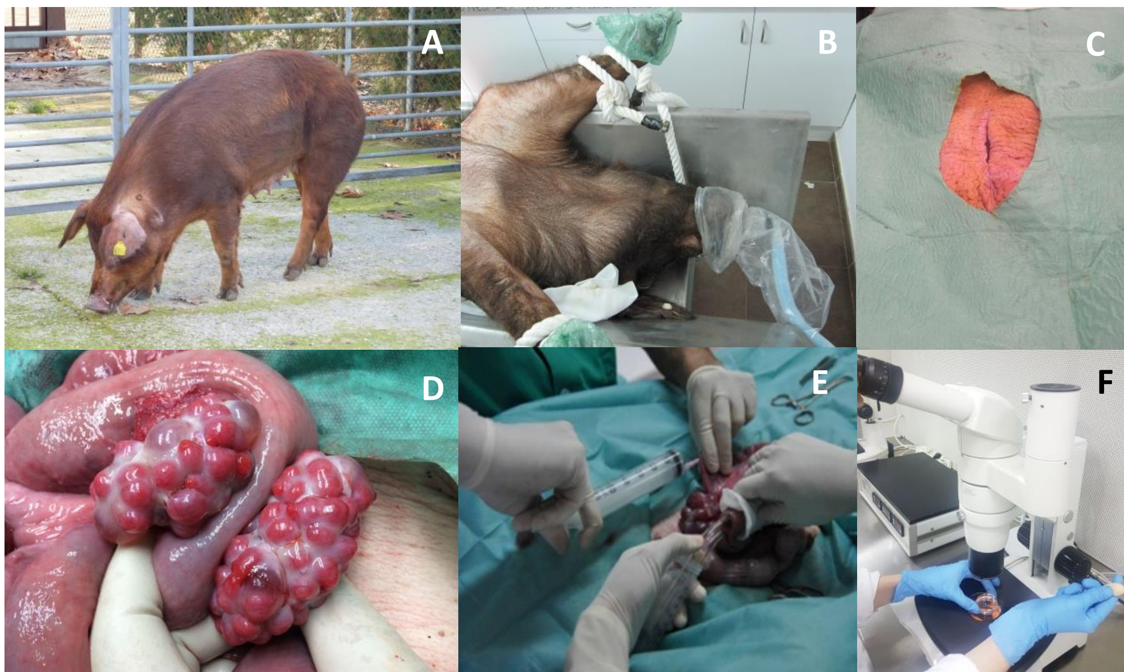

Figure 6

Embryo collection

A, Donor sow. B, General anaesthesia was maintained with isofluorane. C, A mid-line incision to expose the reproductive tract. D, Corpora lutea in each ovary were counted. E, Embryos were collected by flushing the tip of each uterine horn with 30 mL of embryo recovery medium. F, Recovery medium was placed into a Petri dish and embryos were located and evaluated using a stereomicroscope to verify their developmental stage and quality.

Figure 7

Vitrification and shipping dewars

A, Straws were submerged in liquid nitrogen, *Super Open pull straw detail. B, Liquid nitrogen tanq. C, Dry shipper dewar.

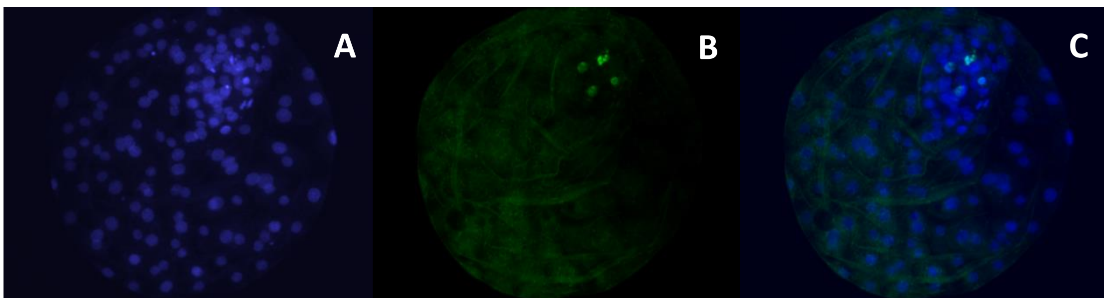

Figure 8

TUNEL assay

A, Vitrified blastocyst stored in a dry shipper for three days and stained with Hoechst. B, TUNEL staining of the same vitrified blastocysts. C, Merded.

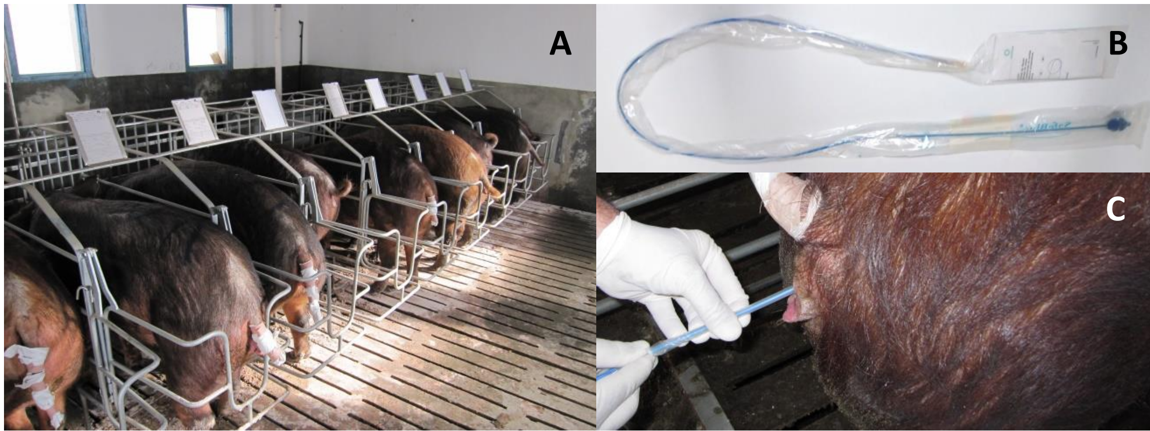

Figure 9

Non-surgical embryo transfer.
A, Detail of a non-surgical embryo transfer catheter. B, Sows were housed individually in crates with mechanically ventilated confinement in the recipient farm. C, Insertion of the non-surgical embryo transfer catheter.

APPENDIX

Journal Profile: REPRODUCTION IN DOMESTIC ANIMALS

Journal Citation Report : Impact factor

JCR Year	AGRICULTURE, DAIRY & ANIMAL SCIENCE		
	Rank	Quartile	Impact factor
2016	**16/58**	**Q2**	**1.400**
2015	19/58	Q2	1.210
2014	13/57	Q1	1.515
2013	15/52	Q2	1.177
2012	13/54	Q1	1.392
2011	15/55	Q2	1.356

Journal Profile: THERIOGENOLOGY

Journal Citation Report : Impact factor

JCR Year	VETERINARY SCIENCES		
	Rank	Quartile	Impact factor
2016	**15/136**	**Q1**	**1.986**
2015	**18/138**	**Q1**	**1.838**
2014	20/133	Q1	1.798
2013	17/132	Q1	1.845
2012	12/143	Q1	2.082
2011	16/145	Q1	1.963

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Journal Profile: Animal

Journal Citation Report : Impact factor

JCR Year	VETERINARY SCIENCES		
	Rank	Quartile	Impact factor
2016	**16/136**	**Q1**	**1.921**
2015	**15/138**	**Q1**	**2.056**
2014	19/133	Q1	1.841
2013	19/132	Q1	1.784
2012	28/143	Q1	1.648
2011	25/145	Q1	1.744

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