UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

DEPARTAMENTO DE MEDICINA Y CIRUGÍA ANIMAL

Optimización del sistema de fecundación *in vitro* en la especie porcina: Condiciones de maduración y de cocultivo de los gametos

Optimization of porcine in vitro fertilization: maturation and gamete coculture conditions

Carmen Almiñana Brines

2008

UNIVERSIDAD DE MURCIA

Departamento de Medicina y Cirugía Animal

FACULTAD DE VETERINARIA
G.I. "Reproducción Animal"
Campus Universitario de Espinardo
30071 MURCIA

Emilio A. Martínez García y María Antonia Gil Corbalán, Profesores del Departamento de Medicina y Cirugía Animal de la Universidad de Murcia

AUTORIZAN

La presentación de la Tesis Doctoral titulada **"Optimización del sistema de fecundación in vitro en la especie porcina: Condiciones de maduración y de cocultivo de los gametos"** realizada por Dña. Carmen Almiñana Brines, bajo nuestra inmediata dirección y supervisión, en el Departamento de Medicina y Cirugía Animal y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

Para que conste a los efectos oportunos, emitimos este informe en Murcia a 23 de Octubre de 2007.

UNIVERSIDAD
DE MURCIA
DPTO. DE MEDICINA Y CIRUGÍA ANIMAL
Facultad de Veterinaria

Dª. Ana Maria Montes Cepeda, Profesora Titular de Universidad del Área de Medicina y Cirugía Animal y Director del Departamento de Medicina y Cirugía Animal, INFORMA:

Que la Tesis Doctoral titulada "Optimación del sistema de fecundación in vitro en la especie porcina:Condiciones de maduración y de cocultivo de los gametos", ha sido realizada por Dª Carmen Almiñana Brines, bajo la inmediata dirección y supervisión de D. Emilio Arsenio Martínez Garcia y Dña. Antonia Gil Corbalán, y que el Departamento ha dado su conformidad para que sea presentada ante la Comisión de Doctorado.

Murcia, a 30 de octubre de 2007

UNIVERSIDAD DE MURCIA
Dpto. Medicina y Cirugía Animal
FACULTAD VETERINARIA

Campus Universitario de Espinardo • 30071 Murcia (España) • Teléf. y Fax: 968 36 47 37

TESIS DOCTORAL COMO COMPENDIO DE PUBLICACIONES

1. Gil MA, Almiñana C, Cuello C, Parrilla I, Roca J, Vazquez JM, Martinez EA. 2007. Brief coincubation of gametes in porcine in vitro fertilization: Role of sperm:oocyte ratio and post-coincubation medium. *Theriogenology.* 67; 620-626.

2. Almiñana C, Gil MA, Cuello C, Roca J, Vazquez JM, Martinez EA. 2005 Adjustments in IVF system for individuals: value of additives and time of sperm-oocyte co-incubation. *Theriogenology.* 64; 1783-1796.

3. Almiñana C, Gil MA, Cuello C, Parrilla I, Roca J, Vazquez JM, Martinez EA. 2007. Effects of ultrashort gamete co-incubation on porcine in vitro fertilization. *Anim. Reprod. Sci.* doi:10.1016/j.anireprosci.2007.05.017

4. Almiñana C, Gil MA, Cuello C, Caballero I, Roca J, Vazquez JM, Martinez EA. 2007. In vitro fertilization (IVF) in straws and a short gamete coincubation time improves the efficiency of porcine IVF. *Reprod. Domestic. Anim. doi:10.1111/j.1439-0531.2007.00995.x.*

5. Almiñana C, Gil MA, Cuello C, Caballero I, Roca J, Vazquez JM, Martinez EA. 2007. In vitro maturation of porcine oocytes with retinoids improves embryonic development. *Reprod. Fertil. Dev.* (en revision).

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Emilio A. Martínez García y Maria Antonia Gil Corbalán, Profesores del Departamento de Medicina y Cirugía Animal de la Universidad de Murcia

AUTORIZAN

A la Licenciada en Veterinaria Dña. Carmen Almiñana Brines a presentar la Tesis Doctoral titulada **"Optimización del sistema de fecundación in vitro en la especie porcina: Condiciones de maduración y de cocultivo de los gametos"** 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 Veterinaria. La Tesis es un compendio de 5 artículos publicados en revistas internacionales de gran difusión en el mundo de la Biotecnología de la Reproducción incluidas en el JCR. Este formato permite plasmar con facilidad el recorrido realizado por la doctoranda, 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 18 de Septiembre de 2007.

UNIVERSIDAD DE MURCIA

Departamento de Medicina y Cirugía Animal

FACULTAD DE VETERINARIA
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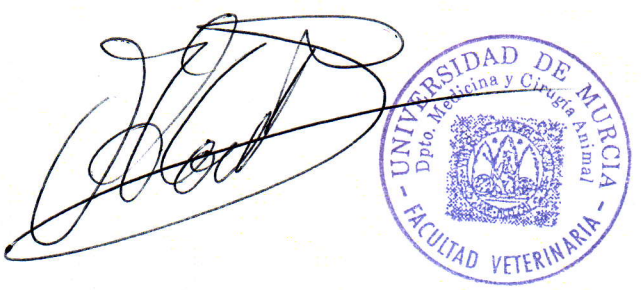
30071 MURCIA

Dª. Ana María Montes Cepeda, Profesora Titular de Universidad del Área de Medicina y Cirugía Animal y Director del Departamento de Medicina y Cirugía Animal,

AUTORIZA:

A la Licenciada en Veterinaria Dña. Carmen Almiñana Brines a presentar la Tesis Doctoral titulada **"Optimización del sistema de fecundación in vitro en la especie porcina: Condiciones de maduración y de cocultivo de los gametos"** ante la comisión de Doctorado como compendio de Publicaciones dado que cumple con todos los requisitos exigidos por la normativa vigente. Dicha Tesis ha sido realizada bajo la inmediata dirección y supervisión de D. Emilio Arsenio Martínez García y Dña. Maria Antonia Gil Corbalán.

Para que conste a los efectos oportunos, emitimos este informe en Murcia a 18 de Septiembre de 2007.



UNIVERSIDAD
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VICERRECTORADO DE ESTUDIOS

D.ª CARMEN ALMIÑANA BRINES
C/ Gabriel Hernández, 45
46760 - Tavernes de la Valldigna
VALENCIA

Vista la solicitud presentada el día 18 de septiembre de 2007 por D.ª Carmen Almiñana Brines, con DNI número 20030007, sobre autorización para presentación de tesis doctoral como compendio de publicaciones con carácter previo a la tramitación de la misma en la Universidad de Murcia, le comunico que la Comisión de General de Doctorado, vistos:

- El informe previo del Departamento de Medicina y Cirugía Animal, responsable de la autorización de la tesis doctoral en fase de elaboración, de esta Universidad, y
- El visto bueno de la Comisión de Grupo de Áreas de Ciencias de la Salud,

resolvió, en su sesión de 19 de octubre de 2007, **ACCEDER** a lo solicitado por el interesado pudiendo, por lo tanto, presentar su tesis doctoral en la modalidad de compendio de publicaciones.

Lo que en cumplimiento del artículo 58 de la vigente Ley 30/1992, de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común, de 26 de noviembre, se **notifica a** D.ª Carmen Almiñana Brines, significándole que contra esta resolución, que pone fin a la vía administrativa, se podrá interponer potestativamente ante el mismo órgano que la ha dictado, recurso de reposición, en el plazo de un mes a contar desde el día siguiente a su notificación, de acuerdo con lo dispuesto en el art. 116 de la citada Ley.

Si no hiciera uso del recurso de reposición podrá interponer recurso contencioso-administrativo, en el plazo de dos meses desde la notificación de este acuerdo, en la forma establecida en la Ley 29/1998, de 13 de julio, reguladora de dicha Jurisdicción.

Murcia, 19 de octubre de 2007
Vicerrectora de Estudios y
Presidenta de la Comisión General de Doctorado

Concepción Palacios Bernal

UNIVERSIDAD DE MURCIA
Vicerrectorado de Estudios

The University Of Sheffield.

School Of Medicine & Biomedical Sciences.

To Whom It May Concern

Professor A P Weetman, Dean

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25th October 2007

Dear Sir / Madam

I have studied Ms. **Carmen Almiñana Brines** doctoral thesis entitled "Optimization of porcine in vitro fertilization: maturation and gamete coculture conditions".

The scientific work presented in the thesis addresses a current problem in the field of animal biotechnology. Although it is now several years that we have the capability of producing porcine embryos in vitro, so far the efficiency of porcine embryo production has been very poor in compare to other livestock species. The scientific work presented in this thesis has highlighted some of the points that can be optimised to further improve efficiency of porcine in vitro fertilization. The data presented are not only helpful to the field of animal biotechnology, but can be used in improving the Human assisted reproduction procedures.

In my opinion this thesis represents a cohesive scientific work that has made important and valuable contributions to the improvement of in vitro fertilization process in porcine species. The thesis is organised in to several chapters that all (except one) is already published in respected peer reviewed international scientific journals. In addition to the science presented, I found the artistic layout of the thesis very interesting too. One particular attractive and motivating point was the progression of the chapters from an immature oocyte to the developed embryo!

I have no hesitation to recommend this thesis for a doctoral degree.

Yours faithfully,

Alireza Fazeli

WAGENINGEN UNIVERSITY
WAGENINGEN UR

To whom it may concern

Adaptation Physiology

DATE
9 November 2007

HANDLED BY
Dr.ing. W. Hazeleger

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I have reviewed the work performed by for the PhD thesis entitled: Optimización del sistema de fecundación *in vitro* en la especie porcina: Condiciones de maduración y de cocultivo de los gametos. This study concerns the optimization of in vitro fertilization procedures (Ch 1-4) and optimization of in vitro maturation of porcine oocytes (Ch 5). The optimization procedures of in vitro fertilization has been directed to prevent polyspermic penetration, an important hurdle in porcine IVF procedures. This has been done by reducing incubation period of sperm with oocytes and optimizing culture conditions. Additionally differences in sperm quality between boars have been adressed by optimizing conditions per boar, indicating the importance of taking in account boar differences in IVF procedures. The optimization procedure for oocyte maturation (Ch 5) added additional value in the development of optimized IVF procedures. This thesis is an important contribution to the international efforts to develop and optimize IVF procedures in pigs. The studies have been performed by well designed experiments, which are presented and discussed in a clear way, with a right choice of bibliography. Most chapters are already published in Theriogenology (2), Animal Reproduction Science and Reproduction in Domestic Animals. The last paper has been submitted to Reproduction, Fertility and Development. The acceptance of these manuscripts by these journals indicates additionally the importance and scientific quality and value of this research. To my opinion the thesis of Miss Carmen Almiñana has for sure the scientific level as required for an European Doctorate without any reservations from my side.

Yours sincerely,

Dr. W. Hazeleger

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II
DIPARTIMENTO DI SCIENZE DEL SUOLO, DELLA PIANTA, DELL'AMBIENTE E DELLE PRODUZIONI ANIMALI

I REVIEWED THE WORK PERFORMED BY 'CARMEN ALMINANA BRINES' IN VIEW OF HER DOCTORAL THESIS ENTITLED "*OPTIMIZACIÓN DEL SISTEMA DE FECUNDACIÓN IN VITRO EN LA ESPECIE PORCINA: CONDICIONES DE MADURACION Y DE COCULTIVO DE LOS GAMETOS*"

THIS DIFFICULT TASK HAS BEEN FACED UNDER FIVE DIFFERENT APPROACHES, SYNTHESIZED IN FIVE DIFFERENT PAPERS AS FOLLOWS:

Paper n.1)
M.A. Gil , C. Almin˜ana, C. Cuello, I. Parrilla, J. Roca, J.M. Vazquez, E.A. Martinez.
Brief coincubation of gametes in porcine in vitro fertilization: Role of sperm:oocyte ratio and post-coincubation medium. Theriogenology 67 (2007) 620–626
Paper n. 2)
C. Almin˜ana, M.A. Gil, C. Cuello, J. Roca, J.M. Vazquez,H. Rodriguez-Martinez , E.A. Martinez.
Adjustments in IVF system for individual boars: Value of additives and time of sperm–oocyte co-incubation.
Theriogenology 64 (2005) 1783–1796
Paper n. 3)
C. Almi˜nana, M.A. Gil, C. Cuello, I. Parrilla, J. Roca, J.M. Vazquez, E.A. Martinez .
Effects of ultrashort gamete co-incubation time on porcine *in vitro* fertilization.
Animal Reproduction Science (2007) (ACCEPTED FOR PUBLICATION)
Paper n. 4)
C Almin˜ ana, MA Gil, C Cuello, I Caballero, J Roca, JM Vazquez and EA Martinez.
In Vitro Fertilization (IVF) in straws and a short gamete coincubation time improves the efficiency of porcine IVF.
Reprod Dom Anim (ACCEPTED FOR PUBLICATION)
Paper n. 5)
C Almiñana, MA Gil, C Cuello, I Caballero, J Roca, JM Vazquez, E Gomez, EA Martinez. In vitro maturation of porcine oocytes with retinoids improves embryonic development. (SUBMITTED)

THE PAPERS PROVIDE SUBSTANTIAL CONTRIBUTION TO THE OPTIMIZATION OF THE IVF SYSTEMS FOR IN VITRO PIG EMBRYO PRODUCTION, WITH SPECIAL EMPHASIS TO THE SPERM-OOCYTE INTERACTION AND COINCUBATION TIMES; OF PARTICULAR INTEREST IS THE IDEA TO USE RETINOIDS FOR IMPROVING EFFICIENCY IN EMBRYO DEVELOPMENT, WHICH-HOWEVER- REQUIRES FURTHER WORK.
ALL THE PAPERS HAVE BEEN VERY WELL CONCEIVED AND PERFORMED AND THE RESULTS ARE VERY CLEARLY ILLUSTRATED.
PRESENTATION IS EXCELLENT AND BIBLIOGRAPHY EXHAUSTIVE.
TO MY OPINION, THIS THESIS IS OF GREAT INTEREST FOR SCIENTISTS AND BREEDERS ENGAGED IN PIG PRODUCTION, AND REACHES THE QUALITY LEVEL REQUIRED FOR AN EUROPEAN DOCTORAL THESIS.

Prof. Dino Di Berardino
Chair of Animal Breeding and Genetics
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Ihr Zeichen / Ihre Nachricht vom
vom

Unser Zeichen / Unsere Nachricht
Datum

2007-11-29

Evaluation Report

of the Thesis from:

Mrs. Carmen Almiñana Brines

entitled:

Optimization of porcine in vitro fertilization: maturation and gamete co-culture conditions

Current knowledge:
In vitro production of porcine embryos has been developed in principle more 30 years ago. However, despite of intensive research some critical steps of in vitro maturation, in vitro fertilization and in vitro culture have not been solved sufficiently. Among these are incomplete maturation of the ooplasm, polyspermic fertilization and reduced embryo developmental competence.
As matured oocytes and competent embryos as a prerequisite for many new biotechniques like cloning, transgenesis and stem cell research, research on in vitro production of porcine embryos is continuing worldwide.

Objectives of the present thesis:
The present thesis is in the focus of this research and investigates the problem of polyspermic fertilization. The experiments have already been published in international journals or are under editorial review. The papers are entitled:

- Brief coincubation of gametes in porcine in vitro fertilization: Role of sperm:oocyte ratio

and post-coincubation medium.
- Adjustments in IVF system for individual boars: Value of additives and time of sperm–oocyte co-incubation.
- Effects of ultra-short gamete co-incubation time on porcine in vitro fertilization.
- In Vitro Fertilization (IVF) in Straws and a Short Gamete Coincubation Time Improves the Efficiency of Porcine IVF.
- In vitro maturation of porcine oocytes with Retinol improves embryonic development.

Results:
The results of the five experiments indicate that in vitro production of porcine embryos can be improved and procedures may be simplified. The key information is:

- Depending on the sperm –oocyte ratio, the coincubation time can be reduced from more than 6h to 10 minutes. Independently, it takes 2 h before spermatozoa penetrate the oocyte. (Experiment 1).
- Further shortage of coincubation to as little as 2 minutes are still sufficient for fertilization. However, the shorter coincubation time has no beneficial effect on the rate of monospermic fertilization. (Experiment 3)
- Polyspermic fertilization is not reduced when caffeine is replaced by HA and/or Adenosine. Individual boar effects seem to overwrite possible advantages of the medium supplements. (Experiment 2)
- An obviously positive effect was seen when in vitro fertilization was performed in a plastic straw. Employing 10 minutes incubation time allowed broadening the sperm-oocyte ratio without increasing polyspermy rates. (Experiment 4)
- Retinol added to the maturation medium seemed to have a positive effect on embryo development and could help to improve embryo survival rates. (Experiment 5)

Overall judgment:

The thesis is well written. Its structure is well organised and very informative. Experimental design and statistical analysis as well as the interpretation of the results in the discussion are very good and match the standards of international research. The project fits well into the objectives of the research group in Murcia and contributes to the merrits of the university.

Altogether the thesis has been excellently performed and fulfils the requirements of a European PhD Thesis very well.

Mariensee 29.11.2007

D. Rath

Prof. Dr.med.vet.habil.Detlef Rath

Este trabajo ha sido financiado por la Fundación Séneca (00197/BPS/04), (04543/GERM/07), el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (RZ01-019) y por el Ministerio de Ciencia y Tecnología (AGL2004-07546, CDTI 04-0231).

A Marco

A mi familia

Agradecimientos

A mi director de tesis, el Dr. Emilio Martínez le dedico mis primeras palabras, por brindarme la oportunidad de iniciarme en la investigación, por contagiarme" el gusanillo" por este trabajo, que tan hondo me ha llegado. Por su apoyo, comprensión y paciencia, que tanto han significado para mi. Por lo mucho que me ha enseñado, el tiempo dedicado y por inculcarme el valor de un trabajo cuidado y bien realizado. Para mi es un orgullo poder trabajar a su lado.

A mi directora de tesis, la Dra. Maria Antonia Gil Corbalán, por sus enseñanzas, su dedicación y su infinita paciencia en mis comienzos, por acogerme en el laboratorio y comprender a aquella ignorante principiante que se emocionaba al ver "sus primeros blastocistos". Gracias por descubrirme este "mundo de ovocitos y embriones" que tanto me apasiona y muchas gracias por ser mi guía en este trabajo.

A los Dr. Jordi Roca y Juan Maria Vázquez, que han sido parte importante de mi formación, no solo en lo académico, sino también en lo humano. Gracias por su apoyo y sus enseñanzas durante estos años, por ser todo un ejemplo para mí de lo que gustaría llegar a ser algún día.

A la Dra. Xiomara Lucas, la primera persona que me abrió la puerta de este departamento en aquella "entrevista de alumnos internos", y por la que siento verdadera admiración. Gracias por su cariño, comprensión y por sus consejos siempre que los he necesitado.

A Nacho, mi compañero de fatigas, por su apoyo incondicional, por soportar "la terrible pesadilla" que he podido llegar a ser, sobretodo durante este último año. Gracias por animarme y mimarme, por compartir tristezas, risas e innumerables cervezas, por acompañarme en el camino para conseguir mi meta.

A Cristina, por las innumerables horas que hemos compartido dentro y fuera del laboratorio, quien diría que "mis bichitos" iban a unir tanto. Gracias por cuidarme y por estar a mi lado siempre que la he necesitado, a veces como compañera, a veces como madre y siempre como una gran amiga.

A mis compañeros de Departamento, por lo mucho que me habéis enseñado cada uno de vosotros, por ayudarme a crecer en todos los sentidos. A Marta, por mostrarme su afán por saber y querer aprenderlo todo como nadie, a Tere por ponerle chispa y alegría a cualquier momento de la vida, a Inma por ser mi maestra en "aquello de los puntos" en mis comienzos y por su capacidad de trabajo, a Eva por ser un claro ejemplo de que con empeño todo se consigue, Jonatan por demostrarme lo fácil que es "hacer amigos" en nuestro taller de sondas, a Mª Luisa, Mª Dolores y Toñi Murcia, a todos ellos, gracias por todos los momentos compartidos, por las interminables sobremesas, sin vosotros me hubiera sido imposible sentirme tan a gusto en el departamento, mi segunda casa. A los recién llegados, a Carolina, porque con ella he disfrutado enseñándole "mi mundo de bichitos" y me ha hecho descubrir "mi lado docente". A Kassia por compartir conmigo interminables tardes en el departamento y acompañarme en la recta final de este trabajo

.

A mis "compis" de piso, a las que llegaron primero y a las que lo hicieron al final, a Olga, M ª Carmen, Laura, Eva, por escucharme cada noche, por soportar mis eternas conversaciones sobre trabajo, por comprender mis escasa horas de convivencia, por vuestra compañía, por las mil palabras de ánimo en los momentos difíciles. Por la amistad que nos une después de tantos años y que espero, que a pesar del tiempo y la distancia perduren para siempre.

A la Fundación Séneca y la Empresa Castillo Larache S .L , por la concesión de mi beca de investigación que han hecho posible la realización de este trabajo.

A las Industrias Fuertes-El Pozo S.A por facilitarme el material biológico, si el cual no podría haber realizado estas experiencias.

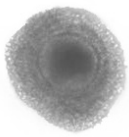
Finalmente, a mi familia, por ser el soporte de mi vida, porque cada uno de ellos con su ejemplo me ha demostrado que nada es imposible. A mis hermanas, por soportarme tantas y tantas cosas, porque cada una a su manera sé que siempre está a mi lado, porque nunca les he dicho lo especiales que son para mí y que cada una representa una parte que no consigo encontrar en mi misma. A mis padres, a quienes admiro, gracias por darme las mejores armas para triunfar en la vida, y por hacer de mí la persona que soy. En especial a mi madre, por ser el mejor ejemplo de fortaleza y de lucha, porque tu fuerza me acompaña día a día. Después de todo llegué al final con "mis bichitos", lo conseguí, aunque no estés a mi lado para verlo, espero que al menos te sientas orgullosa de mí, porque para mí siempre serás la mejor.

A Marco, como siempre "el último"pero imprescindible en mi vida, mi mejor complemento. El que me impulsa a seguir en los momentos difíciles, a saltar las "enormes vallas" que van apareciendo en mi camino, a luchar por conseguir mis sueños. Gracias por estar siempre conmigo aunque la distancia nos separe, por abrirme los ojos y ayudarme a comprender las cosas desde otra perspectiva, fuera de" mi mundo burbuja". Por esa forma tan sutil de hacerme ver mis errores y por ayudarme a levantarme cada vez que he tropezado, por ser el único capaz de hacerme sonreír en los peores momentos de mi vida y porque a tu lado me siento grande y capaz de todo en la vida.

Y ya está. Después de unos años este es el fruto de mi pasión y esfuerzo. Gracias a todos los que me apoyaron en conseguir esta meta tan importante en mi vida. A todos ellos los dedico esta tesis, porque el éxito de este trabajo es tanto mío como vuestro, Muchas Gracias de corazón.


Índice

Índice



Introducción

Introducción 1

Durante los últimos años la biotecnología de la reproducción ha experimentado un gran desarrollo, aportando nuevas técnicas con gran repercusión en el campo de la producción ganadera y la salud humana. Cabe destacar el creciente interés que despiertan en la actualidad las investigaciones sobre clonación, transgénesis y células madre embrionarias. Estas tecnologías permiten el estudio de la regulación génica del desarrollo y ofrecen una esperanza para el tratamiento de graves enfermedades en la especie humana. Tanto la transgénesis como la clonación requieren el empleo de ovocitos maduros y, en determinados casos, zigotos pronucleares monospérmicos. Este hecho ha llevado a los laboratorios de fecundación *in vitro* (FIV) a intentar perfeccionar los sistemas de maduración (MIV), FIV y cultivo *in vitro* (CIV) con el fin de obtener una fuente de embriones para la aplicación de estas biotecnologías. Entre las distintas especies en las que se aplica la producción *in vitro* (PIV) de embriones cabe destacar la especie porcina por sus aplicaciones en biomedicina, ya que se considera modelo para la especie humana por sus semejanzas fisiológicas y especie de elección para los xenotransplantes.

A pesar de los numerosos avances llevados a cabo durante los últimos años en el campo de la MIV, FIV y CIV embriones en la especie porcina, aún no se ha conseguido un procedimiento que proporcione tasas aceptables de desarrollo embrionario. Las principales razones para este bajo rendimiento son: la baja calidad de los ovocitos madurados *in vitro* (Funahashi y cols., 1997; Nagai, 2000; Kikuchi y cols., 2002), la elevada tasa de polispermia (Niwa, 1993; Abeydeera y Day, 1997a; Wang y cols., 1997; Abeydeera, 2002; Gil y cols., 2004a, b, 2007; Almiñana y cols., 2005, 2007a, b), las bajas tasas de desarrollo embrionario (Abeydeera y Day, 1997a; Abeydeera, 2001; Kikuchi y cols., 2002) y la pobre calidad embrionaria de los ovocitos producidos o cultivados *in vitro* (Wang y cols., 1999; Han y cols., 1999a, b; Kikuchi y cols., 2002; McCauley y cols., 2003).

Como se ha mencionado anteriormente, entre las numerosas causas que pueden motivar la baja tasa de desarrollo embrionario, la alta incidencia de polispermia es quizás la más importante. A pesar de los muchos estudios realizados con el fin de reducir la alta tasa de polispermia en esta especie, la incidencia de penetración polispérmica en los ovocitos porcinos fecundados *in vitro* continúa siendo extremadamente elevada comparada con otras especies (Niwa, 1993; Funahashi y Day, 1997; Prathers y Day, 1998; Day, 2000; Funahashi, 2003). Esta elevada incidencia de penetración polispérmica observada en todos los sistemas de FIV porcina podría ser debida a un bloqueo de zona inadecuado o retrasado en los ovocitos madurados in vitro y/o a las condiciones que rodean a los gametos durante la FIV (revisado por Abeydeera, 2002). Aunque los embriones polispérmicos pueden desarrollarse *in vitro* hasta el estadio de blastocisto (Han y cols., 1999a, b), e incluso iniciar una gestación al ser transferidos a cerdas receptoras, su desarrollo completo es inviable, lo que causa la elevada mortalidad embrionaria y/o fetal tras la transferencia de embriones producidos *in vitro*.

Se han descrito diversos factores que afectan a la penetración espermática y a la tasa de polispermia en la especies porcina, tales como el medio de cocultivo (Fraser, 1995; Abeydeera y Day, 1997b; Martínez-Madrid y cols., 2001; Kidson y cols., 2001) el ratio de espermatozoide:ovocito (Rath, 1992; Xu y cols., 1996; Gil y cols., 2004a), la fuente de espermatozoides (Wang y cols., 1994; Abeydeera y Day, 1997a; Gil y cols., 2005), el sistema de FIV (Funahashi y Nagai, 2000; Beebe y cols., 2002; Li y cols., 2003; Clark y cols., 2002, 2003, 2005) y el tiempo de coincubación de los gametos (Coy y cols., 1993; Ocampo y cols., 1994; Abeydeera y Day, 1997a; Marchal y cols., 2002; Gil y cols., 2004b).

Diferentes autores han señalado que la reducción del periodo de coincubación de los espermatozoides con los ovocitos durante la FIV porcina podría ser un método efectivo para reducir la polispermia y mejorar la eficiencia de la FIV (Cheng y cols., 1986; Mattioli y cols., 1989; Abeydeera y Day, 1997a). Abeydeera y Day (1997a) tras realizar el cocultivo de los gametos desde las 3 a las 12 h, observaron que el 31% de los ovocitos habían sido penetrados a las 3 h después de la FIV, alcanzando la máxima penetración a las 6 h. Además, demostraron un incremento significativo de la tasa de polispermia al extender el tiempo de cocultivo de las 6 a las 12 h. En la actualidad, la mayoría de laboratorios que trabajan en FIV porcina han reducido el tiempo de coincubación de 12-18 h, usadas en los primeros sistemas de FIV (Cheng y cols., 1986; Mattioli y cols., 1989), a 5-6 h (Abeydeera y Day, 1997a; Suzuki y cols., 2000; Gil y cols., 2003). Recientemente, se ha propuesto disminuir aún más el tiempo de coincubación, de las 5 h a los 10 min,

con el fin de minimizar el número de espermatozoides presentes en el medio de fecundación (Grupen y Nottle, 2000), en un intento de reducir la polispermia y mejorar la eficiencia de la PIV porcina. Los resultados obtenidos hasta ahora con este nuevo sistema por diferentes laboratorios (Grupen y Nottle, 2000; Gil y cols., 2004b) son contradictorios. La mayor parte de los protocolos que emplean tiempos cortos de coincubación de los gametos comparten la práctica común de lavar los ovocitos mediante pipeteo mecánico en medio limpio de FIV con el fin de eliminar los espermatozoides no adheridos a la zona pelúcida, transfiriendo los ovocitos una vez lavados a otra gota de medio de FIV (sin espermatozoides) e incubarlos hasta que se completan las tradicionales 5-6 h de coincubación. Se ha demostrado que los espermatozoides que quedan adheridos a la zona pelúcida en los primeros 10 min de coincubación son capaces de fecundar posteriormente un alto número de ovocitos (Grupen y Nottle, 2000; Gil y cols., 2004b; Funahashi y Romar, 2004; Almiñana y cols., 2005). Además, se sabe, que la penetración espermática en los ovocitos porcinos madurados *in vitro* ocurre ya a las 2 h post-inseminación (Hunter y Dziuk, 1968; Marchal y cols., 2002). Por lo tanto, una posible forma de aumentar la eficiencia *in vitro* de un tiempo corto de coincubación podría ser el uso de periodos de post-incubación en un medio no adecuado para la FIV. Por otro lado, sería necesario establecer el ratio de espermatozoides:ovocito adecuado para estos sistemas ya que es sabido que este parámetro es uno de los factores más importantes que condicionan la alta tasa de polispermia (Abeydeera y Day, 1997a). Por todo ello, nuestro primer objetivo fue evaluar el efecto de diferentes ratios espermatozoide:ovocito durante la FIV utilizando un corto tiempo de coincubación (10 min) y diferentes periodos de post-coincubación en un medio no apropiado para la FIV sobre los parámetros de fecundación.

La adición de ciertas sustancias al medio de coincubación de gametos, ha sido la base de diversos estudios sobre fecundación *in vitro* con el objetivo de minimizar la alta incidencia de polispermia. En la mayoría de sistemas de FIV porcina los espermatozoides están expuestos a cafeína (Yoshida, 1987; Nagai y cols., 1988; Mattioli y cols., 1989; Wang y cols., 1991; Funahashi y Day, 1993), la cual es conocida por su capacidad de estimular la motilidad (Garbers y cols., 1973) y, presuntamente, la capacitación (Wang y cols., 1991; Funahashi y cols., 2000a) y la reacción acrosómica espontánea (RAE) (Funahashi y cols., 2000b). Sin embargo, se ha sugerido que la RAE inducida por la cafeína podría estar relacionada con la alta incidencia de polispermia (Funahashi y Nagai, 2001). Recientemente, la penetración polispérmica se ha visto ampliamente reducida al reemplazar la cafeína por la adenosina en el medio de fecundación (Funahashi y cols., 2000a, b; Funahashi y Nagai, 2001). En estos estudios, el análisis de la capacidad

funcional de los espermatozoides expuestos a este aditivo, reveló que la adenosina estimula la capacitación pero inhibe la RAE. Por otro lado, el ácido hialurónico (AH) tiene una acción similar a la adenosina, ya que parece inducir la capacitación del espermatozoide sin la posterior RAE (Rodríguez–Martínez y cols., 1998). Además, no hay que olvidar que el AH podría jugar un papel importante en la modulación de las penetraciones polispérmicas *in vivo*, como sugiere su presencia en el fluido intraluminal del oviducto porcino (Tienthai y cols., 2001) y su secreción por los complejos cúmulus-ovocitos (COCs) durante la MIV (Suzuki y cols., 2000; Yokoo y cols., 2002). A pesar de los estudios mencionados anteriormente, sólo unos pocos experimentos, con resultados dispares, se han realizado con el fin de evaluar el efecto *in vitro* del AH sobre la incidencia de la polispermia en la especie porcina (Suzuki y cols., 2000 y 2002). Más aún, ninguno de ellos ha evaluado el efecto conjunto de estos suplementos durante la coincubación de los gametos sobre la fecundación polispérmica. Otro factor que afecta a las tasas de penetración y polispermia en los programas de FIV porcina es la variación individual entre machos. Dicha variabilidad es un hecho constatado en esta especie y está ampliamente aceptada por los laboratorios de FIV, tanto cuando se usa semen fresco como congelado (Wang y cols., 1991; Sirard y cols., 1993, 1995; Suzuki y cols., 1994). Estas diferencias considerables entre verracos sugieren que todos los machos no responden de igual forma a las mismas condiciones de FIV. Por lo tanto, es necesario definir las mejores condiciones *in vitro* para los espermatozoides de cada individuo con el fin de alcanzar un protocolo adecuado que pueda ofrecer bajos porcentajes de polispermia de forma reproducible y repetitiva. El segundo objetivo fue aumentar la eficiencia de FIV porcina optimizando un protocolo de FIV para machos individuales. Las diferencias entre verracos fueron evaluadas según la respuesta de los espermatozoides a la FIV usando como aditivos en el medio de fecundación la cafeína, el AH y la adenosina. Teniendo en cuenta la importancia del tiempo de coincubación expuesta anteriormente, en este trabajo se emplearon diferentes tiempos de coincubación de los gametos.

Es interesante señalar que en la mayoría de los muchos y diversos protocolos utilizados en los laboratorios de FIV los ovocitos están expuestos durante un largo periodo de tiempo a un alto número de espermatozoides. Sin embargo, se ha demostrado que la penetración *in vitro* de los ovocitos porcinos así como la fecundación polispérmica ocurren ya a las 2-3 h post-inseminación cuando se utilizan espermatozoides congelados/descongelados (Abeydeera y Day, 1997a; Marchal y cols., 2002; Gil y cols., 2007) o espermatozoides frescos (Martínez y cols., 1996). Además, se sabe que la incidencia de espermatozoides vivos bajo condiciones de capacitación, permanece constante después de 2, 4, y 6 h de coincubación con los ovocitos (Vázquez y cols.,

1993). Esto sugiere que al extender el periodo de coincubación de los gametos, se tiende a aumentar el número de interacciones espermatozoides-ovocito dando lugar a una alta incidencia de penetración polispérmica. Por lo tanto, parece obvio que una larga exposición de los ovocitos a los espermatozoides podría no ser necesaria o incluso podría ser perjudicial. Los hallazgos encontrados en los estudios llevados a cabo en nuestro laboratorio (Almiñana y cols., 2005; Gil y cols., 2007), junto con el hecho de que en la especie humana se han conseguido buenas tasa de penetración y una baja incidencia de polispermia utilizando tiempos ultracortos (30 s) de coincubación (Bungum y cols., 2006), indican que 10 min podría ser todavía un periodo demasiado largo. Por ello, en el tercer objetivo evaluamos el efecto de tiempos de coincubación ultracortos en la eficiencia de la FIV porcina.

Como hemos expuesto anteriormente, aunque las condiciones bajo las cuales se lleva a cabo la FIV difieren entre los distintos laboratorios, un factor común en los todos ellos es la exposición de los ovocitos a un excesivo y no fisiológico número de espermatozoides durante la coincubación de los gametos. Este alto número de espermatozoides por ovocito causa simultánea penetración espermática con la consecuente fecundación polispérmica (Wang y cols., 2003). Además, las condiciones ambientales durante la FIV están lejos de parecerse al ambiente oviductal en el tracto genital de la hembra e ignoran las estrategias que acontecen *in vivo* para la capacitación espermática y la natural selección de los espermatozoides después de la inseminación (Hunter y Rodriguez-Martínez, 2004). Por ello, recientemente se han propuesto nuevas estrategias tales como el sistema de FIV en pajuela (Li y cols., 2003), el método de "climbing-over-wall" (Funahashi y Nagai, 2000), o la tecnología de microfluidos (Beebe y cols., 2002; Clark y cols., 2002, 2003, 2005) tratando de imitar el proceso de fecundación *in vitro* en el tracto oviductal, donde los espermatozoides son gradualmente capacitados durante el transporte al lugar de la fecundación, (Rodríguez-Martínez y cols., 2005), reduciendo la incidencia de fecundación polispérmica (Funahashi y Nagai, 2000). En ese intento de minimizar una masiva exposición de los ovocitos a los espermatozoides, se propuso la combinación de un sistema alternativo a la microgota de FIV junto con un corto tiempo de coincubación. Ya que, es quizás la combinación de estrategias y no el estudio de un factor aislado lo que ayude a mejorar el rendimiento de la FIV, dada la complejidad del entorno y de los eventos que acontecen durante esta. Basándonos en todo lo expuesto hasta ahora, el cuarto objetivo fue evaluar si la combinación de un sistema de FIV en pajuela y un corto tiempo de coincubación (10 min) es una estrategia adecuada para disminuir la fecundación polispérmica y mejorar la eficiencia de la PIV de los blastocistos porcinos.

Además de los factores relacionados directamente con las condiciones de cocultivo de los gametos, la maduración *in vitro* de los ovocitos parece ser clave para los resultados de la FIV. Aunque la obtención de ovocitos maduros *in vitro* es satisfactoria y con un alto nivel de repetibilidad en la mayoría de los sistemas actuales de FIV, el ambiente durante la MIV podría no ofrecer una adecuada maduración citoplasmática y molecular (Sirard y cols., 2006). Esta maduración citoplasmática y molecular es esencial para preparar al ovocito para los eventos post-fecundación, y permite un desarrollo embrionario normal hasta el estadio de blastocisto. Si comparamos los sistemas *in vitro* con la maduración *in vivo*, las condiciones durante la MIV son demasiado simples y materialmente limitadas, lo que puede afectar profundamente a la maduración del ovocito. Por esta razón, una mejora en las condiciones de MIV sería esencial para obtener una adecuada maduración citoplasmática y nuclear del ovocito, que llevaría a una mejora notable en la eficiencia de la PIV de embriones. El retinol y sus metabolitos, all- *trans* retinol (ROH) y 9- *cis* ácido retinoico (RA) han demostrado tener efectos beneficiosos sobre la competencia citoplasmática después de la maduración de los ovocitos bovinos (Duque y cols., 2002; Gómez y cols., 2003; Ikeda y cols., datos no publicados) y el desarrollo y calidad embrionaria (Bortolotto, 2000; Duque y cols., 2002; Hidalgo y cols., 2003; Gómez y cols., 2003; Livingston y cols., 2004; Lima y cols., 2006). Además, estos compuestos intervienen en el crecimiento celular, y diferenciación y desarrollo embrionario *in vivo* e *in vitro* (Shaw y cols., 1995; Hidalgo y cols., 2003). Teniendo en cuenta que hasta el momento no se ha realizado ningún experimento evaluando el efecto del retinol en la especie porcina, estos efectos sugieren que la introducción de los retinoides en el medio de maduración podría mejorar el rendimiento de la PIV porcina. Aunque se ha demostrado el efecto beneficioso del retinol en la PIV de embriones de distintas especies, la concentración parece ser un factor limitante. Una excesiva exposición de los metabolitos del retinol resulta perjudicial para el desarrollo embrionario en ratones y bovino (Sport y Roberts, 1991; Huang y cols., 2006). El quinto objetivo fue evaluar el efecto del retinol y su concentración durante la MIV sobre los parámetros de MIV, FIV y el desarrollo embrionario en la especie porcina.

Objetivos

Objetivos 2

El objetivo fundamental de la presente tesis doctoral fue optimizar el sistema de fecundación *in vitro* en la especie porcina mediante el estudio de distintas condiciones de maduración y de cocultivo de los gametos. Para ello se propusieron los siguientes objetivos específicos:

1. Evaluar el efecto de diferentes ratios espermatozoide:ovocito durante la FIV utilizando un corto tiempo de coincubación (10 min) y diferentes periodos de post-coincubación en un medio no apropiado para la FIV sobre los parámetros de fecundación (Artículo 1).

2. Optimizar un protocolo de FIV para machos individuales, usando como aditivos la cafeína, el ácido hialurónico y la adenosina en el medio de fecundación y diferentes tiempos de coincubación de los gametos (Artículo 2).

3. Examinar el efecto de tiempos de coincubación ultracortos en la eficiencia de la fecundación *in vitro* porcina (Artículo 3).

4. Estudiar si la combinación de un sistema de FIV en pajuela con un corto tiempo de coincubación podría ser una estrategia adecuada para disminuir la polispermia y mejorar la producción de blastocistos porcinos *in vitro* (Artículo 4).

5. Determinar el efecto de la administración de dos metabolitos del retinol, all-*trans* retinol (ROH) y 9- *cis* ácido retinoico a diferentes concentraciones durante la maduración *in vitro* sobre los parámetros de FIV y el desarrollo embrionario (Artículo 5).

Artículo 1

Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 67 (2007) 620–626
Theriogenology
www.theriojournal.com

Brief coincubation of gametes in porcine in vitro fertilization: Role of sperm:oocyte ratio and post-coincubation medium

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Received 25 May 2006; received in revised form 5 September 2006; accepted 17 September 2006

Abstract

In this study, a short coincubation time of 10 min was used to determine the effect of different sperm:oocyte ratios during in vitro fertilization (IVF), and different periods of post-coincubation in a medium that is not appropriate for IVF, on fertilization parameters. In the first experiment, a total of 1624 in vitro matured oocytes, from 4 replicates, were inseminated with frozen–thawed spermatozoa at different sperm:oocyte ratios (2000, 1500, 1000 and 500 sperm:oocyte) and coincubated for 10 min or 6 h. The oocytes from 10 min of coincubation were washed in IVF medium to remove spermatozoa not bound to the zona pellucida and transferred to another droplet of the same medium (containing no spermatozoa) for 6 h. The oocytes from the other group remained with the spermatozoa for 6 h. Oocytes from both groups were then cultured in embryo culture medium (IVC) for 12 h to assess fertilization parameters. In the second experiment, 1872 in vitro matured oocytes, in 3 replicates were inseminated with frozen–thawed spermatozoa using the same sperm:oocyte ratios as in the first experiment. The oocytes were coincubated for 10 min and transferred directly to IVC medium for 18 h (group A), to IVF medium (containing no sperm) only for 2 h and then to IVC medium for 16 h (group B), or to IVF medium (containing no sperm) for 6 h and then to IVC medium for 12 h (group C or control). There was an effect of sperm:oocyte ratio on all fertilization parameters in experiment 1. The efficiency of IVF (number of monospermic oocytes/total number inseminated) was higher ($P < 0.05$) for oocytes coincubated with spermatozoa for 10 min and inseminated with 1500 and 1000 sperm:oocyte (35.8 ± 3 and 37.6 ± 2.7%, respectively) and for those coincubated for 6 h with 500 spermatozoa per oocyte (37.2 ± 3.1%). In experiment 2, the penetration and efficiency rates obtained in group A were poor (between 3 and 15%) irrespective of the sperm:oocyte ratio. However, in group B the fertilization parameters were similar to the controls and were also affected by the sperm:oocyte ratio. These results demonstrate that coincubation time may be reduced to 10 min to increase the efficiency of fertilization depending on the sperm:oocyte ratio, and that the spermatozoa bound to the zona pellucida require a maximum of 2 h in an appropriate medium to penetrate the oocytes.

Keywords: Coincubation time; Porcine; In vitro fertilization; Oocyte

1. Introduction

In spite of all attempts reported in the literature, the incidence of polyspermic penetration in porcine oocytes fertilized in vitro is still extremely high compared with that in other species [1–5]. This abnormality is due to inadequate and delayed establishment of the in vitro matured zona block and/or the conditions involved

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0093-691X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2006.09.022

during in vitro fertilization (IVF) (revised by Abeydeera [6]). Conditions such as the coculture medium [7–10], sperm:oocyte ratio [11–13], source of spermatozoa [14–16] and coincubation time [15,17–20] affect the sperm penetration and subsequently the polyspermy rate. In this regard, the reduction of gamete coincubation time during IVF from 5 h to 10 min, maintaining the oocytes with the zona-bound spermatozoa in fresh fertilization medium without spermatozoa for an additional 5 h incubation period, has been proposed as a possible strategy to increase the efficiency of in vitro pig embryo production [20–23]. However, this short time of coexposure of the gametes has lead to contradictory results among laboratories that cannot be explained in terms of an increase in penetration rate [21] or a high incidence of polyspermy [20]. In addition, it is well known that polyspermy is related to the absolute number of spermatozoa in the coculture site [15]. Therefore, it can be presumed that the effectiveness of the use of short periods of coincubation during IVF to decrease polyspermy depends on the sperm:oocyte ratio.

All short coincubation times reported in previous studies shared the common practice of washing the oocytes by mechanical pipetting in fresh IVF medium to remove spermatozoa not bound to the zona, transferring the washed oocytes to another droplet of IVF medium (containing no spermatozoa) and incubating them until 5–6 h of a normal IVF coincubation time are completed. However, it has been shown that spermatozoa bound to the zona within the first 10 min are able to fertilize a high number of oocytes [20–23]. It is also known that sperm penetration of in vitro matured porcine oocytes occurs as early as 2 h post-insemination [19,24]. Therefore, a possible way to increase the efficiency in vitro of short coincubation times could be to use post-coincubation periods in media that are not suitable for IVF.

The aim of this study was, using a short coincubation time of 10 min, to evaluate the effect of different sperm:oocyte ratios during IVF, and different periods of post-coincubation in a medium not appropriate for IVF, on in vitro fertilization parameters.

2. Materials and methods

2.1. Reagents and culture media

All chemicals used in this study were purchased from Sigma–Aldrich Co. (Alcobendas, Madrid, Spain) unless otherwise stated.

The basic medium used for the collection and washing of oocytes–cumulus complexes was Dulbecco's phosphate-buffered saline medium supplemented with 4 mg/mL BSA (fraction V; 8022, initial fractionation by cold alcohol precipitation), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 μg/mL kanamicyn (mDPBS). The oocyte maturation medium was the BSA-free North Carolina State University 23 composed of 108.73 mM NaCl, 4.78 mM KCl, 1.7 mM $CaCl_2 \cdot 2H_2O$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7H_2O$, 25.07 mM $NaHCO_3$, 5.55 mM D-glucose, 1 mM L-glutamine, 7 mM taurine, 5 mM hypotaurine, 75 μg/mL potassium penicillin G and 50 μg/mL streptomycin sulfate [NCSU-23; [25]] and supplemented with 10% (v:v) porcine follicular fluid, 0.1 mg/mL cysteine, 10 ng/mL epidermal growth factor, 10 IU/mL eCG (Folligon, Intervet International B.V., Boxmeer, The Netherlands) and 10 IU/mL hCG (Chorulon, Intervet International B.V., Boxmeer, The Netherlands). The basic medium used for IVF was the same as that used by Abeydeera and Day [8] designated as modified Tris-buffered medium (mTBM) and consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (Trizma Base), 11 mM D-glucose and 5 mM sodium pyruvate and supplemented with 2 mM caffeine and 0.2% BSA (fraction V; A-7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was NCSU-23 with 0.4% BSA (fraction V; 8022, initial fractionation by cold alcohol precipitation).

2.2. In vitro oocyte maturation, fertilization and embryo culture

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% (w:v) NaCl containing 70 μg/mL kanamycin and maintained at 25–30 °C. Oocytes were aspirated from medium-sized follicles (3–6 mm in diameter) with an 18-g needle fixed to a 10 mL disposable syringe. Oocytes surrounded by a compact cumulus mass and having evenly granulated cytoplasm were washed three times in maturation medium and 45–50 oocytes were transferred into each well of a Nunc 4-well multidish containing 500 μL of pre-equilibrated maturation medium, previously covered with warm mineral oil and cultured at 39 °C in 5% CO_2 in air for 22 h with added hormones and for another 22 h without hormones. The cumulus cells were then removed with 0.1% hyaluronidase in maturation medium and the denuded oocytes were washed three times and placed in 50 μL drops of pre-equilibrated IVF medium covered with warm mineral oil in a 35 mm × 10 mm petri dish (30 oocytes/drop). The dishes with the oocytes were

kept in the incubator for about 30 min until spermatozoa were added for fertilization.

One pool of two straws of the same mature Pietrain boar tested by his in vitro fertilizing potential and cryopreserved as described by Roca et al. [26] was thawed in a circulating water-bath at 37 °C for 20 s in each replicate. The frozen–thawed sperm characteristics were 53% of motility (CASA system) and 56% of viable spermatozoa with intact acrosome [27]. Just after thawing, 100 μL of spermatozoa were washed three times by centrifugation at 1900 × *g* for 3 min in mDPBS. At the end of the washing procedure, the sperm pellet was resuspended in fertilization medium. After appropriate dilution, 50 μL of this sperm suspension was added to the medium that contained oocytes. Oocytes were coincubated with spermatozoa at 39 °C in an atmosphere of 5% CO_2. For IVC, presumptive zygotes were washed and transferred (30 oocytes/well) to a Nunc 4-well multidish containing 500 μL of embryo culture medium and cultured at 39 °C, 5% CO_2 in air.

2.3. Assessment of fertilization parameters

Eighteen hours after insemination, presumptive zygotes were mounted on slides, fixed for 48–72 h in 25% (v:v) acetic acid in ethanol at room temperature, stained with 1% lacmoid in 45% (v:v) acetic acid, and examined under a phase-contrast microscope at ×400 magnification. Oocytes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei with their corresponding sperm tails and two polar bodies. The fertilization parameters evaluated were: penetration (percentage of the number of penetrated oocytes/total inseminated), monospermy (percentage of the number of monospermic oocytes/total penetrated), number of penetrated spermatozoa/oocyte (mean number of spermatozoa in penetrated oocytes) and efficiency of fertilization (number of monospermic oocytes/total inseminated). Degenerated oocytes, immature oocytes and oocytes with a broken oolemma or abnormal appearance of the cytoplasm were not counted.

2.4. Experimental design

2.4.1. Experiment 1

A total of 1624 in vitro matured oocytes, from 4 replicates, were inseminated with frozen–thawed spermatozoa at different sperm:oocyte ratios (2000, 1500, 1000 and 500 sperm:oocyte or 1.2×10^6, 9×10^5, 6×10^5 and 3×10^5 spermatozoa/mL, respectively) and coincubated for 10 min or 6 h in a 4 × 2 factorial experiment. The oocytes from 10 min of coincubation were washed by mechanical pipetting three times in fresh IVF medium to remove spermatozoa not bound to the zona pellucida, transferred to another droplet and incubated in IVF medium (containing no sperm) for 6 h. The oocytes from the other group remained with the spermatozoa for 6 h. Then all presumptive zygotes were washed and transferred to embryo culture medium for 12 h and fixed and stained to assess fertilization parameters.

2.4.2. Experiment 2

A total of 1872 in vitro matured oocytes, from three replicates, were inseminated with frozen–thawed spermatozoa at different sperm:oocyte ratios (2000, 1500, 1000 and 500 sperm:oocyte) and coincubated for 10 min. Then the oocytes were washed by mechanical pipetting three times to remove spermatozoa not bound to the zona pellucida, and transferred as follows:

(1) directly to IVC medium for 18 h (group A);
(2) to fresh IVF medium, containing no sperm, only for 2 h and then washed and transferred to IVC medium for 16 h (group B);
(3) to fresh IVF medium containing no sperm, incubated for 6 h, washed and transferred to IVC medium for 12 h (group C, control).

All presumptive zygotes were fixed and stained to assess fertilization parameters.

2.5. Statistical analysis

Data were analyzed using SPSS, Version 11.5, software package (SPSS Inc., Chicago, III) and differences were considered significant at $P < 0.05$. Analysis of variance (MIXED procedure) was used to evaluate the effect of the sperm:oocyte ratio, coincubation time and the interaction of both parameters (experiment 1), and the effect of the sperm:oocyte ratio, incubation media and the interaction of both variables (experiment 2) on fertilization parameters. In all cases the random effect of replicate was included in the analysis. Penetration and monospermic rates, and efficiency data, were modelled according to the binomial model of parameters as described by Fisz (1981) before analysis. When analysis of variance showed a significant effect, values were compared using the Bonferroni test. Results are expressed as mean ± S.E.M.

3. Results

3.1. Experiment 1

There was a significant effect of coincubation time ($P < 0.001$), sperm:oocyte ratio ($P < 0.001$) and interaction between both factors ($P < 0.006$) for all fertilization parameters evaluated. A coincubation time of 10 min, compared with 6 h, significantly decreased ($P < 0.005$) sperm penetration in all groups except for those inseminated with 2000 sperm:oocyte (Fig. 1). The decrease in penetration ranged from 10 to 15% for insemination with 1500 and 1000 sperm:oocyte. Insemination with 500 sperm:oocyte and coincubation for 6 h resulted in a similar penetration rate to 2000, 1500 and 1000 sperm:oocyte and 10 min of coincubation. As was expected, the percentage of monospermic oocytes followed an inverse trend compared with the penetration rate; these percentages increased (20–40%) significantly ($P < 0.003$) in all groups of oocytes coincubated with spermatozoa for 10 min. Therefore, the efficiency of IVF, influenced by the penetration and monospermic rates, was between 2.5 and 5 times higher ($P < 0.05$) in the groups of oocytes coincubated with spermatozoa for 10 min and inseminated with 2000, 1500 and 1000 sperm:oocyte, compared with the same groups of oocytes but coincubated with spermatozoa for 6 h. The short coincubation time resulted in a lower mean number of spermatozoa per oocyte compared with oocytes coincubated with spermatozoa for 6 h, except for those inseminated with 500 spermatozoa per oocyte. This sperm number decreased significantly ($P < 0.001$) in a sperm:oocyte ratio-dependent manner only when oocytes were coincubated with spermatozoa for 6 h.

3.2. Experiment 2

There was a significant effect of sperm:oocyte ratio ($P < 0.001$) on penetration, monospermy and number of penetrated spermatozoa per oocyte. The post-coincubation medium affected ($P < 0.001$) all fertilization parameters studied, and, there was interaction (at least $P < 0.05$) between post-coincubation medium and sperm:oocyte ratio on penetration, monospermy and efficiency rates. The results are showed in Fig. 2.

The oocytes transferred directly to IVC medium (group A) showed a very low penetration rate and high monospermy independently of the sperm:oocyte ratio. However, in the oocytes incubated in fresh IVF medium for 2 h (group B) the penetration and monospermy rates were similar to the control group (group C) and showed a clear effect of the sperm:oocyte ratio. Therefore, the experimental groups used in this experiment did not

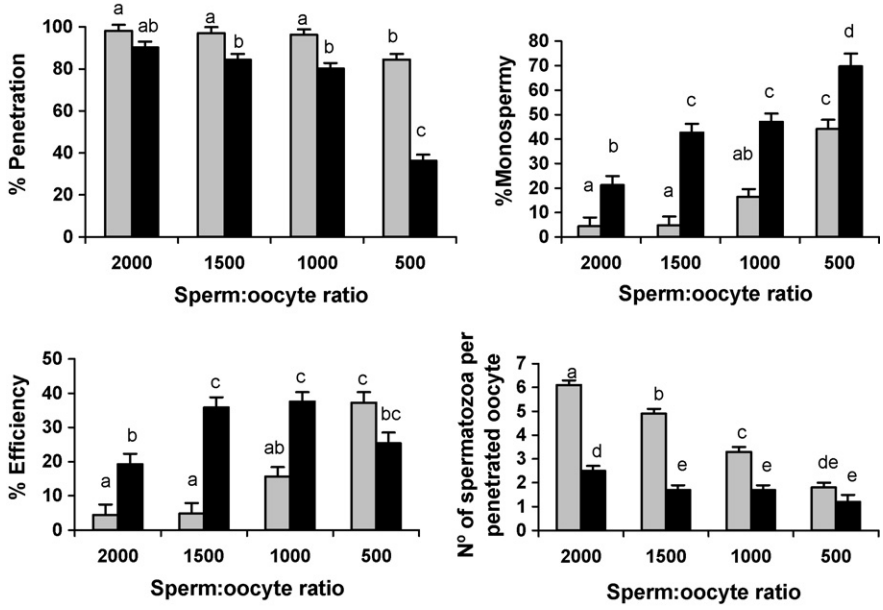

Fig. 1. Effect of sperm:oocyte ratio (2000, 1500, 1000 and 500) and period of co-incubation (( ) 6 h or ( ) 10 min) during in vitro fertilization on the fertilization parameters. Oocytes coincubated for 10 min were washed by mechanical pipetting three times in fresh IVF medium to remove spermatozoa not bound to the zona and transferred to another droplet of mTBM (containing no spermatozoa) until 6 h of insemination was completed. After insemination, presumptive zygotes were washed three times in pre-equilibrated embryo culture medium and cultured for 12 h to assess fertilization parameters. Different letters within the same figure represent a significant difference (at least $P < 0.05$).

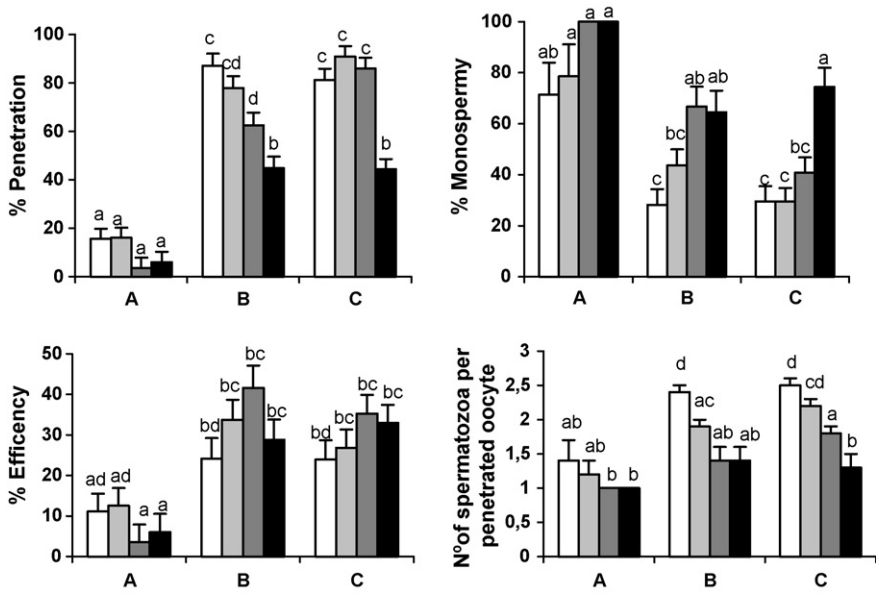

Fig. 2. Effect of the sperm:oocyte ratio ((□) 2000, (▒) 1500, (▓) 1000 and (■) 500) and post-coincubation medium during in vitro fertilization on the fertilization parameters. Oocytes were coincubated with spermatozoa for 10 min, washed by mechanical pipetting three times to remove spermatozoa not bound to the zona pellucida and transferred directly to IVC medium (A); or transferred to fresh IVF medium, containing no spermatozoa for 2 h and transferred to IVC medium (B); or transferred to fresh IVF medium, containing no spermatozoa, incubated for 6 h and transferred to IVC medium (C, control). At 18 h post-insemination all presumptive zygotes were fixed and stained to evaluate fertilization parameters. Different letters within the same figure represent a significant difference (at least $P < 0.05$).

improve the efficiency of fertilization in comparison to the control group.

4. Discussion

A reduction in the period of sperm–oocyte exposure in vitro from 5–6 h to 10 min, maintaining the oocytes in fresh IVF medium without spermatozoa until 6 h, has been suggested as a way to increase the sperm penetration rate of oocytes and the development of IVP embryos to the blastocyst stage [21] or to decrease the incidence of polyspermic penetration [23]. However, when this short coincubation time was used in our laboratory, there were no significant differences in penetration rates compared with long coincubation and, surprisingly, the polyspermy rate increased [20]. The reason for these discrepancies could be attributed, at least in part, to the different source of spermatozoa used in these studies, fresh semen [21,23] versus frozen–thawed sperm [20]. Another reason could be the different boars used, which is supported by another study performed in our laboratory [22]. In that study, although the penetration and polyspermy rates increased significantly with 10 min coincubation for boar A, there were no significant differences between long and short coincubation for any of the fertilization parameters evaluated for boar B. From this work, a boar effect on the fertilizing ability of spermatozoa during short coincubation was evident. Nevertheless, it is necessary to take into consideration others parameters, such as sperm concentration, that could be also implicated in the effectiveness of short coincubation. To confirm this, we investigated in the first part of the present study the influence of different sperm:oocyte ratios (2000, 1500, 1000 and 500) during IVF. There was a clear effect of the sperm:oocyte ratio on the penetration rate, since there were no differences between coincubation times (6 h and 10 min) with 2000 spermatozoa per oocyte but there were with the other sperm:oocyte ratios. Similar to previous studies on short coincubation [20–23], the sperm bound to the zona pellucida within the first 10 min of coincubation were able to fertilize a high proportion of oocytes. However, in this study it was demonstrated that the proportion of oocytes penetrated depended on the sperm:oocyte ratio. Moreover, 6 h of coincubation were required when 500 spermatozoa per oocyte were used, in order to obtain the same penetration rate as that obtained with 2000, 1500 and 1000 spermatozoa per oocyte with only 10 min of coincubation. The monospermic fertilization rate followed an inverse trend to the penetration rate and it was higher in all

groups of oocytes coincubated with spermatozoa for 10 min compared with long coincubation. This result seems to be opposite with the monospermy data obtained in our previous work [20], instead of in both cases boars with high in vitro fertilizing potential have been used. As it has been mentioned above, a boar effect on polyspermic penetration of spermatozoa during the 10 min of coincubation time has been showed [22], therefore, we may thus assume that the monospermy effect of the different sperm:oocyte ratios used in this experiment could have been different with spermatozoa from other boars. As a consequence, the efficiency of fertilization was between 2.5 and 5 times higher with the short coincubation time for high sperm:oocyte ratios (1000–2000) compared with the same groups of oocytes coincubated with spermatozoa for 6 h. Based on these findings, we concluded that the use of a short coincubation time, combined with the high sperm:oocyte ratios used in this study could be a suitable strategy to increase the efficiency of porcine IVF.

In the second experiment, when the oocytes coincubated with sperm for 10 min were washed and transferred directly to IVC medium (group A), the penetration and efficiency rates were poor (between 3 and 15%) irrespective of the sperm:oocyte ratio. Conversely, when the oocytes were washed and maintained in fresh IVF medium without spermatozoa for 2 h (group B), the results were similar to those obtained with the control group of oocytes maintained in fresh IVF medium for 6 h, and they were also influenced by the sperm:oocyte ratio. These results suggest first, that spermatozoa bound to the zona pellucida require a maximum of 2 h in an appropriate medium to penetrate the oocytes, and second, that the IVC medium used in our IVF system (NCSU-23 supplemented with 0.4% BSA) is not suitable for the penetration of oocytes by frozen–thawed spermatozoa. Although the NCSU-23 described by Petters and Wells [25] is very widely used as an in vitro culture medium for pig embryos, there are no data about its effect during IVF. Comparing the NCSU-23 with the IVF medium used in this experiment (mTBM), the mTBM contains a higher Ca^{2+} (7.5 mM), Tris (20 mM) and caffeine concentration (2 mM) and no bicarbonate. It is possible that these differences in composition could explain the low penetration rate of oocytes transferred directly to IVC medium (group A). However, this is questionable as other IVF media contain lower Ca^{2+} and bicarbonate concentrations and, moreover, high penetration rates have been obtained in caffeine-free media [22,28]. Further studies will be needed to explain the low penetration rates when the oocytes with the zona-bound spermatozoa were transferred directly to IVC medium.

In conclusion, the results of the present study showed that with the frozen–thawed spermatozoa used the efficiency of in vitro fertilization was best for a 10 min coincubation combined with high sperm:oocyte ratios (1500 and 1000 sperm:oocyte) and for the long coincubation time combined with the lower sperm:oocyte ratio (500 sperm:oocyte). Post-coincubation of oocytes with the zona-bound sperm in a medium unsuitable for IVF did not improve the efficiency of fertilization. Furthermore, the spermatozoa bound to the zona pellucida required a maximum of 2 h in an appropriate medium to penetrate the oocytes.

Acknowledgements

This study was supported by CICYT-FEDER (AGL2004-07546) and CDTI (04-0231) projects.

References

[1] Niwa K. Effectiveness of in vitro maturation and in vitro fertilization techniques in pigs. J Reprod Fertil 1993;48:49–59.
[2] Funahashi H, Day BN. Advances in in vitro production of pig embryos. J Reprod Fertil 1997;52:271–83.
[3] Prathers RS, Day BN. Practical considerations for the in vitro production of pig embryos. Theriogenology 1998;49:23–32.
[4] Day BN. Reproductive biotechnologies: current status in porcine reproduction. Anim Reprod Sci 2000;60–61:161–72.
[5] Funahashi H. Polyspermic penetration in porcine IVM–IVF systems. Reprod Fertil Dev 2003;15:167–77.
[6] Abeydeera LR. In vitro production of embryos in swine. Theriogenology 2002;57:256–73.
[7] Fraser LR. Ionic control of sperm function. Reprod Fertil Dev 1995;7:905–25.
[8] Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 1997;48:537–44.
[9] Martinez-Madrid B, Domínguez E, Alonso C, Diaz C, Garcia P, Sanchez R. Effect of IVF medium and sperm concentration on fertilization parameters. In: Proceedings of the sixth international conference on pig reproduction; 2001.p. 75.
[10] Kidson A, Colebrander B, Verheijden JHM, Bevers MM. Polyspermy in the pig is dependent on both IVF medium and sperm dose during fertilization in vitro. In: Proceedings of the sixth international conference on pig reproduction; 2001.p. 74.
[11] Rath D. Experiments to improve in vitro fertilization techniques for in vivo matured porcine oocytes. Theriogenology 1992;37:885–6.
[12] Xu X, Seth PC, Harbison DS, Cheung AP, Foxcroft GR. Semen dilution for assessment of boar ejaculate quality in pig IVM and IVF systems. Theriogenology 1996;46:1325–37.
[13] Gil MA, Ruiz M, Cuello C, Vazquez JM, Roca J, Martinez EA. Influence of sperm:oocyte ratio during in vitro fertilization of in vitro matured cumulus-intact pig oocytes on fertilization parameters and embryo development. Theriogenology 2004;61:551–60.

[14] Wang WH, Abeydeera LR, Okuda K, Niwa K. Penetration of porcine oocytes during maturation in vitro by cryopreserved ejaculated spermatozoa. Biol Reprod 1994;50:510–5.
[15] Abeydeera LR, Day BN. Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen–thawed ejaculated spermatozoa. Biol Reprod 1997;57:729–34.
[16] Gil MA, Roca J, Cremades T, Hernandez M, Vazquez JM, Rodriguez-Martinez H, et al. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? Theriogenology 2005;64:305–16.
[17] Coy P, Martinez E, Ruiz S, Vazquez JM, Roca J, Matas C. In vitro fertilization of pig oocytes alter different coincubation intervals. Theriogenology 1993;39:1201–8.
[18] Ocampo MB, Ocampo LC, Mori T, Ueda J, Kanagawa H. Timing of sequential changes in chromosome configurations during the second meiotic division and cytoplasmic events of pig oocytes matured and fertilized in vitro. Anim Reprod Sci 1994;34:281–8.
[19] Marchal R, Pelaez J, Terque M, Mermillod P. Effect of sperm survival and CTC staining pattern on in vitro fertilization of porcine oocytes. Theriogenology 2002;57:1917–27.
[20] Gil MA, Ruiz M, Vazquez JM, Roca J, Day BN, Martinez EA. Effect of short periods of sperm–oocyte coincubation during in vitro fertilization on embryo development in pigs. Theriogenology 2004;62:544–52.
[21] Grupen CG, Nottle MB. A simple modification of the in vitro fertilization procedure. Theriogenology 2000;53:422.
[22] Almiñana C, Gil MA, Cuello C, Roca J, Vazquez JM, Rodriguez-Martinez H, et al. Adjustments in IVF system for individual boars: value of additives and time of sperm–oocyte co-incubation. Theriogenology 2005;64:1783–96.
[23] Funahashi H, Romar R. Reduction of the incidence of polyspermic penetration into porcine oocytes by pre-treatment of fresh spermatozoa with adenosine and a transient co-incubation of the gametes with caffeine. Reproduction 2004;128:789–800.
[24] Hunter RHF, Dziuk PJ. Sperm penetration of pig eggs in relation to the timing of ovulation and insemination. J Reprod Fertil 1968;15:199–208.
[25] Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil 1993;48:61–73.
[26] Roca J, Carvajal G, Lucas X, Vazquez JM, Martinez EA. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen–thawed spermatozoa. Theriogenology 2003;60:77–87.
[27] Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003;68:1828–35.
[28] Funahashi H, Fujiwara T, Nagai T. Modulation of the function of boar spermatozoa via adenosine and fertilization promoting peptide receptors reduces the incidence of polyspermic penetration into porcine oocytes. Biol Reprod 2000;63:1157–63.

Artículo 2

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Theriogenology 64 (2005) 1783–1796
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Adjustments in IVF system for individual boars: Value of additives and time of sperm–oocyte co-incubation

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Received 20 December 2004; received in revised form 14 April 2005; accepted 17 April 2005

Abstract

In vitro fertilization (IVF) in pigs is still considered sub-optimal, due to the variable occurrence of polyspermy, variability mainly related to sperm differences. The present study was conducted in an attempt to increase the efficiency of the in vitro production of porcine embryos by optimizing the in vitro fertilization (IVF) protocol for individual males, with regard to the composition of the fertilization medium (experiments 1 and 2) and the length of gamete co-incubation time (experiment 3). A total of 5943 COC's were in vitro matured (IVM) and inseminated with frozen–thawed spermatozoa from 2 boars (A and B). Experiment 1 determined the effect of additives caffeine (2 mM), hyaluronic acid (HA; [0.5 mg/mL]) and adenosine (10 μM), alone or in combination, to the IVF-medium during sperm–oocyte co-incubation. Experiment 2 tested the addition of various HA (0, 0.5, 1.0 and 1.5 mg/ml) and adenosine (0, 10, 20 and 40 μM) concentrations in the fertilization medium; while experiment 3 investigated the effect of two periods of sperm–oocyte co-incubation (10 min or 6 h). In the case of 10 min sperm–oocyte co-incubation, oocytes with attaching spermatozoa were further cultured in IVF-medium containing no spermatozoa until the 6 h of insemination was completed. Presumptive zygotes were cultured in embryo culture medium for 12–15 h to assess fertilization parameters. In experiment 1, only caffeine significantly influenced the

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0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2005.04.008

outcome of fertilization, albeit being a clearly boar-dependent effect. In experiment 2, similar boar differences were seen for HA supplementation while presence of exogenous adenosine did not influence fertilization parameters in either boar. The results of experiment 3 demonstrated that a short co-incubation time significantly ($P < 0.001$) increased penetration rate and mean number of spermatozoa per oocyte ($74.9 \pm 3.9\%$ versus $62.7 \pm 3.9\%$ and 1.5 ± 3.2 versus 1.3 ± 3.5 for 10 min or 6 h, respectively), but reduced monospermy ($P < 0.001$, $57.9 \pm 2.5\%$ versus $70.0 \pm 2.8\%$) when boar A was used. However, such effects were not seen with boar B, in which sperm–oocyte co-incubation time did not affect the efficiency of fertilization. In view of the present results, a preliminary screening for each individual male is required to select optimal conditions for IVF.

Keywords: Caffeine; Hyaluronic acid; Adenosine; Co-incubation time; In vitro fertilization; Porcine oocytes

1. Introduction

Although extensive attempts have been made to reduce the penetration of the porcine oocytes by more than a single spermatozoon in vitro, the high incidence of polyspermy remains a major impediment in porcine in vitro fertilization (IVF) systems. It is widely accepted that penetration and polyspermy rates in pig IVF are affected by large variations among individual males when fresh or frozen/thawed semen is used [1–4]. These considerable differences among boars suggest that all males do not respond to IVF conditions equally. Therefore, defining the best in vitro conditions for spermatozoa from each individual male is obviously desirable to achieve an optimal IVF system that can provide reproducibly-low rates of polyspermy.

In most current porcine IVF systems [2,5–8] boar spermatozoa are exposed to caffeine, which is known to enhance sperm motility [9] and, presumably, to stimulate capacitation [2,10] and spontaneous acrosome reactions (ARs) [11]. However, it has been suggested that the ARs induced by caffeine may be related to the high incidence of polyspermic fertilization seen [12]. Recently, polyspermic penetration has been greatly reduced by replacing caffeine with adenosine in fertilization medium [10–12]. In those studies, the analysis of the functional state of spermatozoa exposed to this additive revealed that adenosine stimulated capacitation but inhibited spontaneous ARs. Similar to adenosine, hyaluronic acid (HA) appears to induce sperm capacitation without the subsequent ARs seen in its absence [13]. Furthermore, because HA has been localized in the intraluminal fluid of the porcine oviduct [14] and it is secreted by porcine cumulus-oocyte complexes (COCs) during in vitro maturation (IVM) [15,16], HA may play a role during IVF and polyspermy. In spite of the above mentioned studies, only a few, contradictory experiments have been performed in vitro to evaluate the effect of HA on polyspermic fertilization [16,17] and, to the best of our knowledge, no study has reported the combinatorial effect of these supplementations during gamete co-incubation on polyspermic fertilization.

On the other hand, a reduction of the period of sperm–oocyte co-incubation during porcine IVF has also been investigated as an alternative method to reduce polyspermic

fertilization and improve IVF efficiency [7,18–20]. Abeeydera and Day [20] reported that 3 h after IVF, 31% of oocytes were penetrated, with maximum penetration attained by 6 h and without any further increase at 9 or 12 h. On the contrary, such extension of sperm–oocyte co-incubation to 6–12 h resulted in a significant increase in polyspermy rate. Currently, the majority of laboratories working on porcine IVF have reduced the time of gamete co-incubation from the 12–18 h used in earlier porcine IVF systems [7,18], to 5–6 h [17,20,21]. Recently, a new strategy has been proposed to improve the efficiency of in vitro pig embryo production by minimizing the number of spermatozoa present in the fertilization medium [22]. That strategy consisted of decreasing the gamete co-incubation time from 5 h to 10 min, maintaining the oocytes with the zona-bound spermatozoa in fresh fertilization medium, without spermatozoa, for an additional 5 h incubation period. However, when short co-incubation times were tested in our laboratory [23], the penetration rate was not affected, the monospermic rate was reduced, and the overall efficiency of in vitro embryo production did not improve. Because a boar effect could be implicated in these discrepancies, further studies are needed to re-examine the effectiveness of this new strategy.

The present study was designed as an attempt to increase the efficiency of porcine IVF by optimizing an IVF protocol for individual males. Differences between boars were evaluated in response of their spermatozoa to IVF using various additives to the fertilization medium and different intervals of gamete co-incubation.

2. Materials and methods

2.1. Culture media

The medium used for the collection of COCs and washing was Dulbecco's phosphate-buffered saline (DPBS) medium composed of 136.89 Mm NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO4 and 1.46 Mm $CaCl_2{\cdot}2H_2O$ and supplemented with 4 mg/mL bovine serum albumin (BSA; [fraction V]), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 μg/mL of Kanamycin (mDPBS). The oocyte maturation medium was BSA-free North Carolina State University (NCSU-23) [24] supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.1 mg/mL cysteine and 10 ng/mL of epidermal growth factor (EGF). The basic medium used for fertilization was essentially the same as that used by Abeydeera and Day [20]. This medium, designated as modified Tris-buffered medium (mTBM), consists of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2{\cdot}2H_2O$, 20 mM Tris (crystallized free base) 11 mM glucose, 5 mM sodium pyruvate, and BSA (fraction V; A 7888, initial fractionation by cold alcohol precipitation). The embryo culture media was NCSU-23 supplemented with 0.4% BSA (fraction V; A 8022, initial fractionation by cold alcohol precipitation).

Porcine FF was collected at our laboratories from 3 to 6 mm diameter follicles from ovaries obtained from a slaughterhouse, centrifuged at 1500 × *g* for 30 min (15 °C), filtered through 0.8, 0.45 and 0.22 μm filters and stored at −20 °C until use.

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain).

2.2. Recovery and IVM of cumulus-oocyte complexes

The COCs were obtained from ovaries of pre-puberal gilts at a local slaughterhouse and transported to the laboratory at 34–37 °C within 1 h in 0.9% NaCl containing 70 μg/mL of Kanamycin. After the ovaries were washed three times in NaCl solution, follicles 3–6 mm in diameter were aspirated using an 18-gauge needle connected to a 10 ml disposable syringe. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times in maturation medium and selected for culture. COCs were matured in a 4-well multidish (Nunc, Roskilde, Denmark) containing 150–200 COCs per well in 500 μL of maturation medium supplemented with 10 IU/mL of eCG (Folligon, Intervet International B.V., Boxmeer, The Netherlands) and 10 IU/mL of hCG (Chorulon, Intervet International) for 20–22 h and then for another 20–22 h in maturation medium without hormones. COCs were matured under mineral oil at 39 °C in an atmosphere of 5% CO_2 in air.

2.3. In vitro fertilization

Fertilization was performed in mTBM medium supplemented according to the experimental design.

After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium in a vortex during 2 min, 1660 rounds/min. Oocytes were washed twice in maturation medium and three times in pre-equilibrated fertilization medium. Groups of 50 denuded oocytes were then placed in 50 μL-size drops of fertilization medium in a 35 mm × 10 mm Petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, USA) under mineral oil and held at 39 °C in an atmosphere of 5% CO_2 in air for about 30 min until addition of spermatozoa.

Two Pietrain boars (A and B) were selected from a population of 25 boars with proven fertility and normal semen picture (data not shown) used in previous IVF experiments. Results from these experiments showed a great individual variability in penetration and polyspermic rates when subjected to same IVF protocol. Boars A and B were chose because they had shown the highest differences in penetration and polyspermic rates. The sperm-rich ejaculate fractions from these boars had similar semen characteristics in the 5 weeks preceding the experiments (total spermatozoa per ejaculate $>50 \times 10^9$, motility >80%; morphological abnormalities <6% and acrosome abnormalities <15%). The ejaculate fractions were cryopreserved in plastic medium-straws (0.5 mL) as described by Roca et al. [25]. Thawing was carried out in a circulating water-bath at 37 °C for 20 s. After thawing, sperm evaluation was assessed at 30 min using a pool of two straws from each boar in 3 replicates. Sperm motility (60.2 ± 5.14 and 51.7 ± 2.97%, for boar A and B, respectively) and frequency of acrosome abnormalities (36.0 ± 5.24 and 41.0 ± 3.03, for boar A and B, respectively) did not differ significantly between boars.

For IVF, 150 μL of thawed semen from one pool of two straws of each boar were washed three times by centrifugation at 1900 × *g* for 3 min in mDPBS. Each resulting pellet was resuspended in fertilization medium and after appropriate dilution, 50 μL of each sperm suspension was added to 50 μL of the medium that contained the oocytes. The spermatozoa:oocyte ratio was 1000:1. Gametes were co-incubated at 39 °C in humidified

atmosphere of 5% CO_2 and air for 6 h (except in experiment 3 where a co-incubation time of 10 min was also used).

2.4. In vitro culture

At 6 h after insemination, presumptive zygotes were removed from fertilization medium and washed three times in pre-equilibrated embryo culture medium. Subsequently, presumptive zygotes were transferred to a 4-well multidish (50 zygotes per well), each well containing 500 μL of the same medium under mineral oil, and cultured at 39 °C, 5% CO_2 in air for 12–15 h to assess fertilization parameters.

2.5. Assessment of sperm penetration

Oocytes were mounted on slides and fixed in a solution of acetic acid:ethanol (1:3) for 48–72 h at room temperature. Oocytes were then stained with 1% lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of 400×. Oocytes with chromosomes at metaphase-II and an extruded polar body were considered matured. Oocytes were considered penetrated when they had one or more swollen sperm heads and/or male pronuclei, with their corresponding sperm tails present, and two polar bodies. Degenerated oocytes, immature oocytes and oocytes with a broken oolemma or an abnormal appearance of the cytoplasm were not counted.

The fertilization parameters evaluated were: penetration (number of oocytes penetrated/total inseminated), monospermy (number of oocytes containing only one sperm head or one male pronucleus/total penetrated), number of spermatozoa/oocyte (number of spermatozoa in penetrated oocytes), and efficiency of fertilization (number of monospermic oocytes/total inseminated).

2.6. Experimental design

2.6.1. Experiment 1

Two boars (boar A and boar B) were randomly selected to evaluate the effect of caffeine (2 mM), HA (0.5 mg/mL, Bayer, Hyonate vet, Barcelona, Spain) and adenosine (10 μM), alone or in combination, in fertilization medium during sperm–oocyte co-incubation. This experiment was performed using eight different fertilization media: (1) mTBM + caffeine; (2) mTBM + caffeine + HA; (3) mTBM + caffeine + adenosine; (4) mTBM + caffeine + -HA + adenosine; (5) mTBM; (6) mTBM + HA; (7) mTBM + adenosine; (8) mTBM + HA + adenosine. A total of 2042 IVM-oocytes from three replicates (1055 from boar A and 987 from boar B) were inseminated, fixed and stained to assess IVF parameters.

2.6.2. Experiment 2

In an attempt to increase IVF-efficiency using frozen–thawed spermatozoa from the boars described above, various concentrations of HA (0, 0.5, 1.0 and 1.5 mg/mL) and adenosine (0, 10, 20 and 40 μM) were added to the IVF-medium. This experiment was designed according to the necessities of each male, based on results of experiment 1. In this

way, spermatozoa of boar A were washed and introduced into the IVF-medium containing HA or adenosine, but in the absence of caffeine. On the contrary, spermatozoa of boar B were introduced into an IVF-medium with either HA or adenosine, and with caffeine. A total of 1961 IVM-oocytes from three replicates (965 from boar A and 996 from boar B) were inseminated, fixed and stained to assess IVF-parameters.

2.6.3. Experiment 3

In this experiment, the effect of two periods of sperm–oocyte co-incubation (10 min or 6 h) was investigated in each boar, in an attempt to improve the efficiency of IVF. Based on results of experiments 1 and 2, spermatozoa were washed and co-incubated with oocytes for 10 min or 6 h in absence (boar A) or in presence (boar B) of caffeine. Oocytes co-incubated for 10 min were washed three times in fresh fertilization medium using a small pipette to eliminate spermatozoa not attached to the zona pellucida (ZP) and further cultured in IVF-medium (containing no spermatozoa) until the 6 h of insemination was completed. After insemination, presumptive zygotes were washed three times in pre-equilibrated embryo culture medium and cultured for 12–15 h to assess fertilization parameters. A total of 1940 IVM-oocytes from three replicates (945 from boar A and 995 from boar B) were used in this experiment.

2.7. Statistical analysis

All data editing and statistical analysis were performed by SPSS, version 11.5 (SPSS Inc., Chicago, III). Data from three replicates for each boar in each experiment were analyzed by analysis of variance using the MIXED procedure according to a statistical model including the fixed effect of treatment (experiment 1), concentration of Ha or adenosine (experiment 2) or co-incubation time (experiment 3), and the random effect of replicate. Penetration and monospermic rates and efficiency data were modelled according to the binomial model of parameters as described by Fisz [26] before analysis. In experiments 1 and 2, when analysis of variance showed a significant effect, values were compared using the Bonferroni test. The threshold for significance was set to $P < 0.05$. Results are expressed as least squares mean ± S.E.M.

3. Results

3.1. Experiment 1. Effect of caffeine, HA and adenosine during sperm–oocyte co-incubation

As shown in Table 1, addition of caffeine to the IVF medium significantly increased ($P < 0.001$) sperm penetration, polyspermy rate and the mean number of spermatozoa in penetrated oocytes in both boars. However, efficiency rates differed between sires. For boar A, when the fertilization medium contained caffeine, penetration rate was similar among treatments, with values approaching 100%, and monospermy rate was reduced to values between 1.4 and 8.1%. In contrast, in the absence of caffeine, penetration rate was significantly reduced ($P < 0.001$), while the incidence of monospermy increased

Table 1
Effect of caffeine (C), hyaluronic acid (HA) and adenosine (Ad) during sperm–oocyte co-incubation on fertilization parameters in boar A

Treatment							
C	HA	Ad	No. of oocytes	Penetration X ± S.E.M.	Monospermy X ± S.E.M.	EO X ± S.E.M.	Efficiency X ± S.E.M.
+	−	−	135	100 ± 3.8 a	8.1 ± 3.6 a	5.7 ± 3.9 a	17.8 ± 2.7 a
+	+	−	135	98.6 ± 3.8 a	1.4 ± 3.6 a	6.2 ± 3.9 a	16.4 ± 2.7 a
+	−	+	134	100 ± 3.8 a	3.6 ± 3.6 a	6.2 ± 3.9 a	15.3 ± 2.7 a
+	+	+	129	97.7 ± 3.9 a	2.4 ± 3.7 a	6.4 ± 3.9 a	13.8 ± 2.7 a
−	−	−	138	65.3 ± 3.8 b	69.1 ± 4.1 b	1.7 ± 4.1 b	43.3 ± 2.7 b
−	+	−	136	71.2 ± 3.8 b	71.5 ± 4.0 b	1.3 ± 4.1 b	52.6 ± 2.7 b
−	−	+	121	62.7 ± 3.9 b	67.5 ± 4.4 b	1.3 ± 4.2 b	42.1 ± 2.7 b
−	+	+	127	75.5 ± 3.9 b	62.7 ± 4.0 b	1.5 ± 4.1 b	48.8 ± 2.7 b

Different letters within the same column represent a significant difference ($P < 0.05$) at least. Penetration: number of oocytes penetrated/total inseminated, monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated, EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes, efficiency: number of monospermic oocytes/total inseminated.

($P < 0.001$) to 62.7–75.5%. In addition, the efficiency of IVF followed a similar effect to monospermy, decreasing to minimum values (13.8–17.8%) in the caffeine treatments, reaching values between 42.1 and 52.6% in caffeine-free treatments ($P < 0.001$). For boar B, the presence of caffeine was necessary to obtain acceptable rates of penetration and monospermy (Table 2). Contrary to the results observed for boar A, the efficiency of fertilization was higher ($P < 0.001$) when caffeine was present in the IVF-medium (36.2–41.9%), than when caffeine was absent (26.3–30.2%).

On the other hand, under caffeine or caffeine-free conditions, addition of HA, adenosine -or the combination of both to the fertilization medium did not affect the IVF parameters using semen from either boar.

Table 2
Effect of caffeine (C), hyaluronic acid (HA) and adenosine (Ad) during sperm–oocyte co-incubation on fertilization parameters in boar B

Treatment							
C	HA	Ad	No. of oocytes	Penetration X ± S.E.M.	Monospermy X ± S.E.M.	EO X ± S.E.M.	Efficiency X ± S.E.M.
+	−	−	117	64.3 ± 5.0 a	63.9 ± 6.2 ab	1.4 ± 9.1 ab	41.0 ± 4.4
+	+	−	130	73.7 ± 4.8 a	50.6 ± 5.6 a	1.7 ± 8.5 a	36.9 ± 4.1
+	−	+	136	69.8 ± 4.7 a	60.2 ± 6.2 ab	1.4 ± 8.5 ab	41.9 ± 4.0
+	+	+	116	72.3 ± 5.0 a	50.5 ± 5.9 a	1.7 ± 8.8 a	36.2 ± 4.4
−	−	−	118	33.1 ± 4.9 b	79.9 ± 8.0 b	1.2 ± 1.1 b	26.3 ± 4.3
−	+	−	126	36.6 ± 4.8 b	82.8 ± 7.5 b	1.2 ± 1.1 b	30.2 ± 4.2
−	−	+	122	33.6 ± 4.9 b	85.4 ± 7.8 b	1.1 ± 1.1 b	28.7 ± 4.3
−	+	+	122	33.8 ± 4.9 b	83.0 ± 7.8 b	1.2 ± 1.1 b	27.9 ± 4.3

Different letters within the same column represent a significant difference ($P < 0.05$) at least. Penetration: number of oocytes penetrated/total inseminated, monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated, EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes, efficiency: number of monospermic oocytes/total inseminated.

Table 3
Effect of hyaluronic acid (HA) during sperm–oocyte co-incubation on fertilization parameters in boar A

Treatment					
Concentration of HA (mg/ml)	No. of Oocytes	Penetration X ± S.E.M.	Monospermy X ± S.E.M.	EO X ± S.E.M.	Efficiency X ± S.E.M.
0	97	60.8 ± 4.0 a	69.1 ± 6.4	1.4 ± 8.4	42.1 ± 5.6
0.5	134	72.4 ± 3.0 ab	64.9 ± 4.8	1.4 ± 6.3	50.8 ± 4.8
1.0	134	81.3 ± 3.0 bc	70.6 ± 4.6	1.3 ± 6.0	57.4 ± 4.6
1.5	132	90.2 ± 3.0 c	60.5 ± 4.4	1.5 ± 5.7	54.6 ± 4.6

Different letters within the same column represent a significant difference ($P < 0.05$) at least. Penetration: number of oocytes penetrated/total inseminated, monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated, EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes, efficiency: number of monospermic oocytes/total inseminated.

3.2. Experiment 2. Effect of different concentrations of HA and adenosine during IVF

Table 3 shows that for boar A, penetration rate increased ($P < 0.01$) with the addition of HA, in a dose-dependent manner. However, no differences were observed for monospermic rate or the mean number of spermatozoa per oocyte. In this way, the presence of 1 and 1.5 mg/mL of HA showed a tendency ($P < 0.14$) to increase efficiency of fertilization. When the concentrations of HA were tested for boar B, no differences were observed in fertilization parameters (Table 4). A similar pattern was also observed for both boars when the gametes were co-incubated with various concentrations of adenosine. Exposure to adenosine did not influence any of the IVF-parameters (Tables 5 and 6).

3.3. Experiment 3. Effect of sperm–oocyte co-incubation time on fertilization parameters

In boar A, the penetration rate and mean number of spermatozoa per oocyte increased significantly ($P < 0.001$) as the incubation time was decreased (74.9 ± 3.9% versus 62.7 ± 3.9% and 1.5 ± 3.2 versus 1.3 ± 3.5 for 10 min or 6 h, respectively). The proportion of monospermic oocytes was lower ($P < 0.001$) for 10 min (57.9 ± 2.5%) than

Table 4
Effect of hyaluronic acid (HA) during sperm–oocyte co-incubation on fertilization parameters in boar B

Treatment					
Concentration of HA (mg/ml)	No. of Oocytes	Penetration X ± S.E.M.	Monospermy X ± S.E.M.	EO X ± S.E.M.	Efficiency X ± S.E.M.
0	131	62.6 ± 4.2	58.5 ± 5.3	1.6 ± 7.3	36.6 ± 4.3
0.5	122	61.5 ± 4.4	68.0 ± 5.5	1.4 ± 7.6	41.8 ± 4.5
1.0	118	61.9 ± 4.5	68.5 ± 5.6	1.3 ± 7.7	42.4 ± 4.5
1.5	125	64.0 ± 4.3	66.3 ± 5.3	1.4 ± 7.3	42.4 ± 4.4

Penetration: number of oocytes penetrated/total inseminated, monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated, EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes, efficiency: number of monospermic oocytes/total inseminated.

Table 5
Effect of adenosine during sperm–oocyte co-incubation on fertilization parameters in boar A

Treatment					
Concentration of adenosine (μM)	No. of oocytes	Penetration X ± S.E.M.	Monospermy X ± S.E.M.	EO X ± S.E.M.	Efficiency X ± S.E.M.
0	90	61.2 ± 6.7	69.1 ± 6.2	1.4 ± 7.3	42.1 ± 6.2
10	135	63.9 ± 5.9	67.4 ± 5.0	1.4 ± 5.8	43.1 ± 5.2
20	125	61.1 ± 6.0	72.4 ± 5.3	1.3 ± 6.2	44.2 ± 5.3
40	118	64.2 ± 6.1	71.1 ± 5.3	1.5 ± 6.2	45.7 ± 5.5

Penetration: number of oocytes penetrated/total inseminated, monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated, EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes, efficiency: number of monospermic oocytes/total inseminated.

Table 6
Effect of adenosine during sperm–oocyte co-incubation on fertilization parameters in boar B

Treatment					
Concentration of adenosine (μM)	No. of oocytes	Penetration X ± S.E.M.	Monospermy X ± S.E.M.	EO X ± S.E.M.	Efficiency X ± S.E.M.
0	131	62.6 ± 4.2	58.5 ± 5.5	1.6 ± 7.8	36.6 ± 4.3
10	129	65.9 ± 4.2	60.0 ± 5.4	1.5 ± 7.7	39.5 ± 4.3
20	120	66.7 ± 4.3	58.7 ± 5.5	1.5 ± 7.9	39.2 ± 4.5
40	120	68.3 ± 4.3	61.0 ± 5.5	1.4 ± 7.8	41.7 ± 4.5

Penetration: number of oocytes penetrated/total inseminated, monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated, EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes, efficiency: number of monospermic oocytes/total inseminated.

for 6 h (70.0 ± 2.8%) of co-incubation. Nevertheless, sperm–oocyte co-incubation time did not affect the efficiency of IVF (Fig. 1). A different result was obtained for boar B, in which there were no significant differences between 10 min or 6 h of gamete co-incubation, for any of the IVF-variables.

4. Discussion

In recent studies, it has been reported that polyspermic IVM–IVF porcine embryos cleave and develop to the blastocyst stage in vitro at a similar rate to monospermic embryos [27–29]. This fact suggests that evaluating the incidence of embryos that develop to the blastocyst stage is not a suitable measurement for assessing the rate of normal fertilization of porcine embryos. Thus, in the present study the visualization of the pronuclei by using a staining method was used to assess the incidence of normal fertilization of the porcine IVM-oocytes.

Currently, most porcine IVF media are supplemented with caffeine, although it has been reported that caffeine increases the incidence of polyspermic penetration [10,11]. In our study, the use of caffeine had a positive effect on spermatozoa from boar B, showing an increase in the penetration rate and in the efficiency of fertilization. However, in

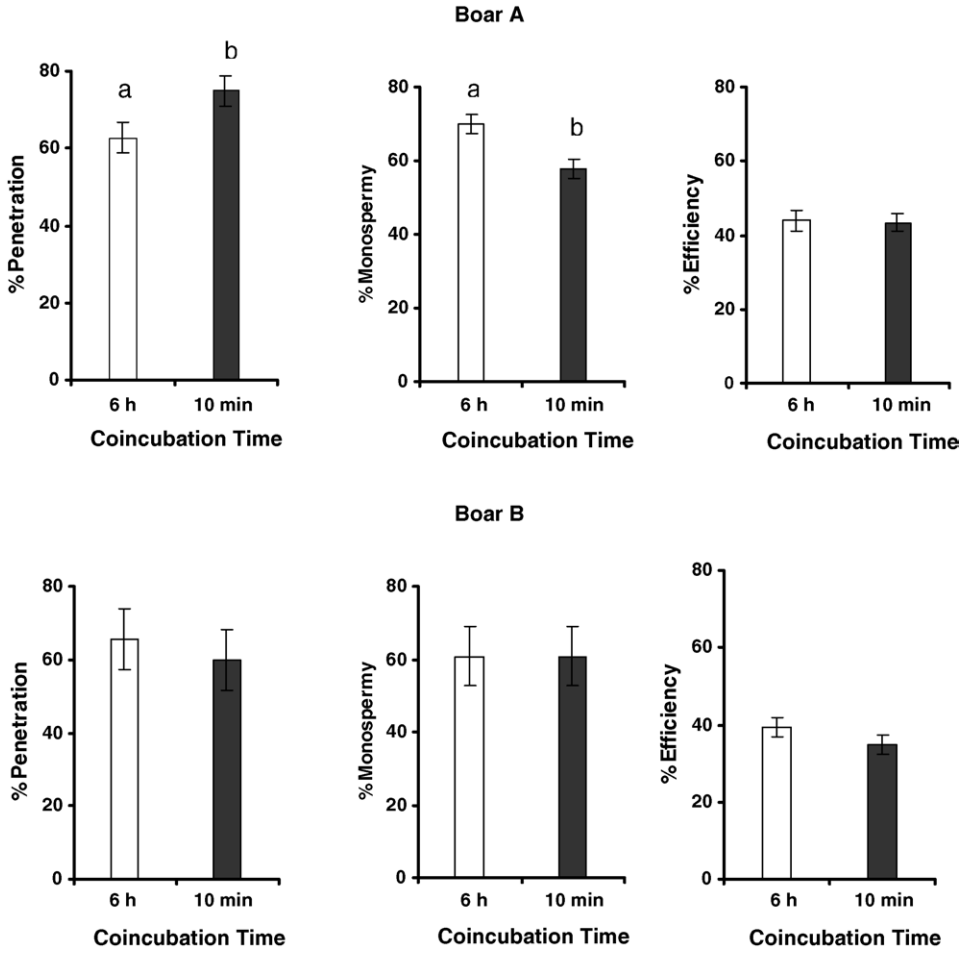

Fig. 1. Effect of two periods of co-incubation time during IVF. Oocytes from three replicates were co-incubated with spermatozoa for 6 h or 10 min in absence (boar A) or presence (boar B) of caffeine. Oocytes co-incubated for 10 min were washed three times in fresh fertilization medium using a small pipette to eliminate spermatozoa not attached to the zona pellucida and further cultured in fertilization medium (containing no spermatozoa) until the 6 h of insemination was completed. After insemination, presumptive zygotes were washed three times in pre-equilibrated embryo culture medium and cultured for 12–15 h to assess fertilization parameters. [a, b] ($P < 0.05$).

spermatozoa from boar A, the obtained effect of caffeine appeared to be detrimental, increasing the incidence of penetration (100%) but also of polyspermic penetration (90%), thus decreasing IVF-efficiency. It is remarkable that frozen–thawed spermatozoa from boar A could penetrate more than 70% of oocytes in the medium without caffeine. These results are in contrast to those obtained by several authors [2,4,30], who reported that boar spermatozoa needed caffeine to penetrate oocytes in vitro, and confirm results from others authors who obtained high rates of penetration of oocytes in caffeine-free medium [31,17,11]. All these findings suggest that there is large variation in the effect of caffeine

upon the rate of sperm penetration and monospermic penetration among semen of different boars. Our results confirm such suggestion. In the present study, the presence of caffeine induced high polyspermic penetration using semen from boar A, while the spermatozoa from boar B needed caffeine to reach an acceptable penetration rate. In other words, caffeine may be a good supplement for IVF for one boar, but not for another.

Culture in the presence of caffeine has been demonstrated to promote not only sperm capacitation but also spontaneous ARs in pigs [10–12]. Moreover, the high incidence of polyspermic penetration is likely due to the simultaneous penetration of a number of spermatozoa with a partially induced AR caused by caffeine in the IVF-medium [12]. Thus, efforts to reduce simultaneous sperm penetration, such as the inhibition of caffeine-induced spontaneous ARs, would regulate the number of spermatozoa capable of fertilizing the oocyte. Following this reasoning, polyspermic fertilization has been reduced by replacing caffeine in the fertilization medium with HA [17] or adenosine [10,11], substances that stimulate capacitation but inhibit spontaneous ARs. In our study, when gametes were co-incubated in presence of HA, no differences were observed in fertilization parameters between the two boars used, regardless of the presence or absence of caffeine in the same medium. This finding is in contrast with results obtained in previous investigations [17], which showed that HA in the IVF-medium improved monospermic penetration without decreasing penetration rate in a conventional porcine IVF system. However, this same research group did not find a clear effect of HA on monospermic penetration [16]. These contradictory results could be explained by the different IVF conditions used (IVF medium, concentration of spermatozoa, sperm:oocyte ratio) in addition to the different boars used as semen donors. In our study, increasing the concentration of HA (experiment 2) from 0.5 to 1.5 mg/mL did not affect monospermic penetration in either boar. However, for boar A, penetration rates significantly increased from 72 to 90%. Overall, these results indicate a poor effect of HA-addition in conventional IVF systems although it would be necessary to confirm it by using a large number of boars.

Recent studies have shown that the addition of adenosine to the fertilization medium not only increased the in vitro penetrability of frozen–thawed boar spermatozoa but also decreased the incidence of polyspermic penetration in porcine oocytes [10,11]. This effect was attributed to the fact that adenosine induces capacitation, but inhibits spontaneous acrosome reactions. In contrast, in our study, when adenosine was added to fertilization medium in the presence or absence of caffeine (experiment 1), and even when different concentrations of this substance were used (experiment 2), no differences were observed in either boar in any of the fertilization parameters in comparison to the control groups. Again, these contradictory results could be explained by the different conditions of IVF systems or by the different boars used in these studies. In our study, the addition of HA and adenosine together, in presence or absence of caffeine, to the fertilization medium neither improved the monospermy rate nor the efficiency of fertilization. Our results suggest that the ARs induced by caffeine may not be as important as suggested in relation to the polyspermic fertilization in pig IVF systems, or that HA and/or adenosine are not able to inhibit the caffeine-induced ARs, at least in the boars used in the present study.

Rates of penetration and polyspermy are also influenced by the duration of the co-incubation of the gametes [review: 32]. Recently, both penetration rate and blastocyst development were improved by exposing the oocytes to the spermatozoa for 10 min

followed by incubating the oocytes with the ZP-bound spermatozoa in fresh fertilization medium for 5 h [22]. However, when this new system was used in our laboratory, the penetration rates were not significantly different and the polyspermy rates were higher compared to those obtained after 6 h of co-incubation [23]. This variability in results could be due to the different boars used in both laboratories since the data obtained in the present study showed an evident boar effect on the fertilizing ability of spermatozoa during different co-incubation times. For boar A, the penetration rate and number of spermatozoa per oocyte increased significantly with the shorter incubation time, but at the expense of a lower monospermy rate. A different result was obtained with boar B, where no significant differences were observed between 10 min or 6 h of co-incubation for any of the fertilization parameters. Despite the different penetration capability with varying co-incubation times, the efficiency of fertilization was similar, regardless of the boar because of the compensatory effect between penetration rate and monospermy. On this point, two considerations are worth of mention. On one hand, high penetration rates can be expected with a co-incubation time as short as 10 min. This statement is supported by the demonstrations that oocyte penetration and polyspermic fertilization occurs as early as 2–3 h post-insemination when frozen–thawed sperm [20,33] or freshly un-diluted or stored (24 h), diluted sperm-rich fraction semen was used [34]. Furthermore, sperm capacitation treatments do not accelerate sperm penetration [34]. Therefore, it seems evident that environmental conditions of co-culture are sufficient to induce capacitation and a true AR, and that spermatozoa are able to interact immediately with oocytes. This hypothesis is supported by data from the present and previous [22,23] studies, which indicate that spermatozoa that bind to the ZP within the first 10 min of co-incubation are able to fertilize a high proportion of oocytes. On the other hand, the reasons for the high incidence of polyspermy obtained at least in some boars, with the short time of co-incubation remain unclear. It is known that the incidence of acrosome-reacted living spermatozoa remain constant after 2, 4, or 6 h of co-incubation with the oocytes [35]. This fact determines that when the sperm–oocyte co-incubation time is extended from 2 to 6 h, the proportion of oocytes penetrated and the number of spermatozoa per oocyte increases gradually over time [34,20] because the possibilities of sperm–oocyte interactions are higher. However, when oocytes are washed after 10 min of co-incubation and then transferred to a medium without spermatozoa, new interactions are prevented, but ZP-bound spermatozoa are not prevented from penetrating the oocytes. Although it has been speculated that lower oxidative stress suffered by the gametes could explain the high incidence of polyspermic penetration observed after short-term co-incubation [23], more research is needed to clarify this phenomenon. A rather likely explanation is that the degree of IVM is not complete and the oocytes are not fully able of eliciting the ZP-reaction after the first spermatozoon has passed, a problem identified as major in porcine IVF [36]. Finally, although no significant differences in sperm quality between boars were detected, the possibility that these results may just be a result of sperm quality (boar A had 10% higher rate of motility and 5% less acrosome abnormalities) cannot be discharged.

In conclusion, the results from the present study indicate that the needs of boar spermatozoa for IVF vary among boars. Among the variables studied (substances add to fertilization medium and co-incubation time), only the presence or absence of caffeine in the IVF-medium had a significant effect on the fertilization efficiency, but with a clear boar

influence. While for boar B the addition of caffeine was necessary to obtain both acceptable penetration and monospermy rates, the presence of caffeine reduced considerably monospermy rates in boar A to less than 10%. However, the efficiency of fertilization was not different when spermatozoa from each boar were appropriately supplemented (boar A without caffeine and boar B with caffeine). Taking into account that the main objective in IVF systems is to reach maximum efficiency of fertilization, preliminary studies for each individual male are required in order to identify the optimal conditions for insemination, because all males do not respond equally to IVF conditions.

Acknowledgments

The authors wish to thank Dr. Tod McCauley for helpful discussion and critical reading of the manuscript. The SENECA foundation, Murcia is acknowledged for funding a guest Professorship for Dr. H. Rodriguez-Martinez. This research was partially supported by INIA (RZ01-019).

References

[1] Sirard MA, Dubuc A, Bolamba D, Zheng Y, Coenen K. Follicule-oocyte–sperm interactions in vivo and in vitro in pigs. J Reprod Fertil 1993;48(Suppl):3–16.
[2] Wang WH, Niwa K, Okuda K. In-vitro penetration of pig oocytes matured in cultured by frozen–thawed ejaculated spermatozoa. J Reprod Fertil 1991;93:491–6.
[3] Wang WH, Abeydeera LR, Fraser LR, Niwa K. Funcional analyisis using chlortetracycline fluorescence and in vitro fertilization of frozen–thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. J Reprod Fertil 1995;104:305–13.
[4] Suzuki K, Ebihara M, Nagai T, Clarke NGE, Harrison RAP. Importance of bicarbonate/CO_2 for fertilization of pig oocyte in vitro, and synergism with caffeine. Reprod Fertil Dev 1994;6:221–7.
[5] Yoshida M. In vitro fertilization of pig oocytes matured in vivo. Jpn J Vet Sci 1987;49:711–8.
[6] Nagai T, Takahashi T, Masuda H, Shioya Y, Kuwayama M, Fukushima M, et al. In vitro fertilization of pig oocytes by frozen boar spermatozoa. J Reprod Fertil 1988;84:585–91.
[7] Mattioli M, Bacci ML, Galeati G, Seren E. Developmental competence of pig oocytes matured and fertilized in vitro. Theriogenology 1989;31:1201–7.
[8] Funahashi H, Day BN. Effects of follicular fluid at fertilization in vitro on sperm penetration in pig oocytes. J Reprod Fertil 1993;99:97–103.
[9] Garbers DL, First NL, Sullivan JJ, Lardy HA. The effects of cyclic nucleotide phosphodiesterase inhibitors on ejaculated porcine spermatozoa metabolism. Biol Reprod 1973;8:599–606.
[10] Funahashi H, Asano A, Fujiwara T, Nagai T, Niwa K, Fraser LR. Both fertilization promoting peptide and adenosine stimulate capacitation but inhibit spontaneous acrosome loss in ejaculated boar spermatozoa in vitro. Mol Reprod Dev 2000;55:117–24.
[11] Funahashi H, Fujiwara T, Nagai T. Modulation of function of boar spermatozoa via adenosine and fertilization-promoting peptide receptors reduce the incidence of polyspermic penetration into porcine oocytes. Biol Reprod 2000;63:1157–63.
[12] Funahashi H, Nagai T. Regulation of in vitro penetration of frozen–thawed boar spermatozoa by caffeine and adenosine. Mol Reprod Dev 2001;58:424–31.
[13] Rodriguez–Martinez H, Larson B, Pertoft H, Kjellen L. GAGs and spermatozoon competence in vivo and in vitro. In: Lauria A, Gandolfi F, Enne G, Gianaroli L, editors. Gamete development and function. Rome: Serono Symposia; 1998. p. 239–72.

[14] Tienthai P, Kjellén L, Pertoft H, Suzuki K, Rodriguez-Martinez H. Localisation and quantitation of hyaluronan and sulphated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. Reprod Fert Dev 2001;12:173–82.
[15] Yokoo M, Tienthai P, Kimura N, Niwa K, Sato E, Rodriguez-Martinez H. Localisation of the hyaluronan receptor CD44 in porcine cumulus cells during in vivo and in vitro maturation. Zygote 2002;10:317–26.
[16] Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodríguez-Martinez H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. Int J Androl 2002;25:84–93.
[17] Suzuki K, Eriksson B, Shimizu H, Nagai T, Rodríguez-Martinez H. Effect of hyaluronanon monospermic penetration of porcine oocytes fertilizated in vitro. Int J Androl 2000;23:13–21.
[18] Cheng WTK. Polge C, Moor RM. In vitro fertilization of pig and sheep oocytes. Theriogenology 1986;25:146.
[19] Coy P, Martinez E, Ruiz S, Vazquez JM, Roca J, Matas C, et al. In vitro fertilization of pig oocytes after different co-incubation intervals. Theriogenology 1993;39:1201–8.
[20] Abeeydera LR, Day BN. Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified Tris-buffered medium with frozen–thawed ejaculated spermatozoa. Biol Reprod 1997;57:729–34.
[21] Gil MA, Abeydeera LR, Day BN, Vazquez JM, Roca J, Martinez EA. Effect of the volume of medium and number of oocytes during in vitro fertilization on embryo development in pigs. Theriogenology 2003;60:767–76.
[22] Grupen CG, Nottle MB. A simple modification of the in vitro fertilization procedure. Theriogenology 2000;53:422.
[23] Gil MA, Ruiz M, Vazquez JM, Roca J, Day BN, Martinez EA. Effect of short period of sperm–oocyte co-incubation during in vitro fertilization on embryo development in pigs. Theriogenology 2004;62:544–52.
[24] Peters RM, Wells KD. Culture of pig embryos. J Reprod Fertil 1993;48:61–73.
[25] Roca J, Carvajal G, Lucas X, Vazquez JM, Martinez EA. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen–thawed spermatozoa. Theriogenology 2003;60:77–87.
[26] Fisz M. Some probability distributions. In: De Krieger R, editor. Probability theory and mathematical statistics. Malabar, FL, USA: John Wiley and Sons; 1980. p. 129–74.
[27] Han YM, Abeeydera LR, Kim JH, Moon HB, Cabot RA, Day BN, et al. Growth retardation of inner cell mass cells in polyspermic porcine embryos produced in vitro. Biol Reprod 1999;60:1110–3.
[28] Han YM, Wang WH, Abeeydera LR, Petersen AL, Kim JH, Murphy C, et al. Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos. Biol Reprod 1999;61:1340–6.
[29] McCauley TC, Mazza MR, Didion BA, Mao J, Wu G, Coppola G, et al. Chromosomal abnormalities in day-6, in vitro produced embryos. Theriogenology 2003;60:1569–80.
[30] Nagai T. Current status and perspectives in IVM–IVF of porcine oocytes. Theriogenology 1994;41:73–8.
[31] Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 1997;48:537–44.
[32] Abeydeera LR. In vitro fertilization and embryo development in pigs. Reproduction 2001;58(Suppl):159–73.
[33] Marchal R, Pelaez J, Terqui M, Mermillod P. Effect of sperm survival and CTC staining pattern on in vitro fertilization of porcine oocytes. Theriogenology 2002;57:1917–27.
[34] Martinez E, Vazquez JM, Matas C, Gadea J, Maria IA, Roca J. Oocyt penetration by fresh or stored diluted boar spermatozoa before and alter in vitro capacitation treatments. Biol Reprod 1996;55:134–40.
[35] Vazquez JM, Martinez E, Roca J, Coy P, Pastor LM. Acrosome reaction of boar spermatozoa in vitro fertilization. Mol Reprod Dev 1993;36:84–8.
[36] Funahashi H, Ekwall H, Kikuchi K, Rodriguez-Martinez H. Transmission electron microscopy studies of the zona reaction in pig oocytes fertilised in vivo and in vitro. Reproduction 2001;122:443–52.

Artículo 3

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Animal Reproduction Science xxx (2007) xxx–xxx

ANIMAL REPRODUCTION SCIENCE

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Effects of ultrashort gamete co-incubation time on porcine *in vitro* fertilization

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Received 21 February 2007; received in revised form 14 May 2007; accepted 16 May 2007

Abstract

A reduction in co-incubation time has been suggested as an alternative method to reduce polyspermic fertilization. The aim of this study was to evaluate the effect of short periods of gamete co-incubation during pig *in vitro* fertilization. A total of 2833 *in vitro* matured oocytes were inseminated with thawed spermatozoa and coincubated for 0.25, 1, 2, 3, 7, 10 min and 6 h. The oocytes from the 0.25–10 min groups were washed three times in modified Tris-buffered medium (mTBM) medium to remove spermatozoa not bound to the zona and transferred to the same medium (containing no spermatozoa) until 6 h of co-incubation time were completed. After 6 h, presumptive zygotes from each group were cultured in NCSU-23 medium for 12–15 h to assess fertilization parameters. After each period of co-incubation, 45–50 oocytes from each group were stained with Hoechst-33342 and the number of spermatozoa bound to the zona was counted. Although the number of zona bound spermatozoa increased ($p < 0.05$) with the co-incubation time, no increase was observed in penetration rates among groups from 2 min to 6 h of co-incubation time (ranging from 53.5 ± 2.8 to $61.3 \pm 2.6\%$). Similarly, the efficiency of fertilization reached a maximum for the 2 min of co-incubation group with values ranging between 32.3 ± 2.4 and $41.9 \pm 2.5\%$. The reduction of co-incubation time did not affect the monospermy rate (range: 71.3 ± 3.4–$80.2 \pm 3.8\%$) and the mean number of spermatozoa/oocyte (range: 1.2 ± 0.4–1.4 ± 0.5). These results show that, under our *in vitro* conditions, high penetration rate can be obtained with co-incubation times as short as 2 min, although monospermy could not be improved using this strategy.

Keywords: Co-incubation time; *In vitro* fertilization; Porcine oocyte

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doi:10.1016/j.anireprosci.2007.05.017

Please cite this article in press as: Almiñana, C. et al., Effects of ultrashort gamete co-incubation time on porcine *in vitro* fertilization, Anim. Reprod. Sci. (2007), doi:10.1016/j.anireprosci.2007.05.017

1. Introduction

Polyspermic penetration has been demonstrated to occur during IVF in different species, but the incidence appears to be extremely high in pigs. Although considerable research has been undertaken trying to solve this problem, the high incidence of polyspermy still remains a persistent obstacle to porcine IVF and often exceeds 50% (Niwa, 1993; Funahashi and Day, 1997; Prather and Day, 1998; Day, 2000; Abeydeera, 2002; Funahashi, 2003).

In most current IVF systems, oocytes are exposed to an excessive and nonphysiologically high number of spermatozoa for a long period. However, it has been reported that penetration *in vitro* of porcine oocytes as well as polyspermic fertilization occur as early as 2–3 h postinsemination with frozen–thawed spermatozoa (Abeydeera and Day, 1997; Marchal et al., 2002; Gil et al., 2007) or with fresh semen (Martinez et al., 1996). Moreover, it is also known that the percentage of true acrosome reacted sperm remained reasonably constant throughout co-incubation (Vazquez et al., 1993). This suggests that extending spermatozoa–oocyte co-incubation times tends to increase spermatozoa–oocyte interactions resulting in a high incidence of polyspermic penetration. Therefore, it seems obvious that a long exposure of oocytes to spermatozoa may be not necessary or may even be harmful.

Currently the period of co-incubation has been reduced from the 12–18 h used in earlier porcine IVF systems (Cheng et al., 1986; Mattioli et al., 1989) to 3–6 h (Coy et al., 1993a; Abeydeera and Day, 1997; Suzuki et al., 2000; Funahashi, 2003; Gil et al., 2003; Funahashi and Romar, 2004) in order to reduce the incidence of polyspermic fertilization. In addition, it has been reported that spermatozoa bind to the zona pellucida (ZP) within the first 10 min of gamete co-incubation and require a maximum of 2 h to penetrate the oocytes (Gil et al., 2007). These spermatozoa are able to fertilize a similar number of oocytes compared to the standard (6 h) co-incubation time (Gil et al., 2004). Although the use of 10 min of co-incubation has been suggested as an alternative method to improve IVF efficiency, the results obtained among laboratories are contradictory in terms of penetration rate, monospermy and efficiency of *in vitro* embryo production (Grupen and Nottle, 2000; Gil et al., 2004; Almiñana et al., 2005; Gil et al., 2007).

Nevertheless, 10 min of gamete co-incubation may still be a period too long, as it has been demonstrated in human IVF, where good fertilization rates and a low incidence of polyspermy can be obtained using ultrashort (30 s) co-incubation (Bungum et al., 2006). Thus, the objective of the present study was to evaluate the effect of ultrashort co-incubation times on the efficiency of porcine IVF.

2. Materials and methods

2.1. Culture media

The medium used for the collection of cumulus-oocyte complexes (COCs) and washing was Dulbecco's phosphate-buffered saline (DPBS) medium composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and 1.46 mM $CaCl_2{\cdot}2H_2O$, and supplemented with 4 mg/ml BSA (fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 μg/mL kanamycin (mDPBS). The oocyte maturation medium was BSA-free North Carolina state university (NCSU-23) (Peters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid, 0.8 mM cysteine and 10 ng/ml EGF. The basic medium used for fertilization was essentially the same as that used by Abeydeera and Day (1997). This medium, designated as modified Tris-buffered medium (mTBM), con-

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sists of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (crystallized free base), 11 mM glucose, 5 mM sodium pyruvate, and supplemented with 0.2% BSA (fraction V; A 7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was NCSU-23, supplemented with 0.4% BSA.

Porcine follicular fluid was collected at our laboratories from 3 to 6 mm diameter follicles from ovaries of prepuberal gilts obtained at a slaughterhouse, centrifuged at $1500 \times g$ for 30 min (15 °C), filtered through 0.8, 0.45, and 0.22 μm filters, and stored at −20 °C until use.

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich Co. (Alcobendas, Madrid, Spain).

2.2. Recovery and in vitro maturation of cumulus-oocyte complexes

Ovaries were obtained from prepuberal gilts at a local slaughterhouse and transported to the laboratory at 34–37 °C within 1 h after collection in 0.9% NaCl containing 70 μg/mL kanamycin. After the ovaries were washed three times in NaCl solution, follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle connected to a 10 ml disposable syringe. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times in maturation medium and selected for culture. COCs were matured in a 4-well multidish (Nunc, Roskilde, Denmark) containing 150–200 COCs per well in 500 μl of maturation medium supplemented with 10 IU/ml eCG (Folligon, Intervet international B.V., Boxmeer, The Netherlands) and 10 IU/ml hCG (Chorulon, Intervet international) for 20–22 h, and then for another 20–22 h in maturation medium without hormones. COCs were matured under mineral oil at 39 °C in an atmosphere of 5% CO_2 in air.

2.3. In vitro fertilization

After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium in a vortex for 2 min, 1660 rounds/min. Oocytes were washed twice in maturation medium and three times in preequilibrated fertilization medium. Groups of 50 denuded oocytes were then placed in 50 μl drops of fertilization medium in a 35 × 10 mm Petri dish (Falcon, Becton Dickinson labware, Franklin lakes, USA) under mineral oil and held at 39 °C in an atmosphere of 5% CO_2 in air for about 30 min until addition of spermatozoa.

Semen from a mature Pietrain boar was processed and cryopreserved in 0.5 mL straws (Minutub Iberica S.L., Tarragona, Spain) as described by Roca et al. (Roca et al., 2003). For each replicate, one pool of semen was made from two straws thawed in a circulating waterbath at 37 °C for 20 s. A preliminary screening was performed for the boar used in order to select the optimal conditions for IVF. So, the IVF medium was not supplemented with caffeine and 1000 sperms per oocyte were used during co-incubation. The characteristics of frozen–thawed spermatozoa evaluated 30 min after thawing were 58 ± 5.0% motility (CASA system, sperm class analyser®, microptic, Barcelona, Spain) and 39.2 ± 5.2% acrosome abnormalities (Pursel and Johnson, 1974). For IVF, 100 μl of thawed semen were washed three times by centrifugation at $1900 \times g$ for 3 min in mDPBS. The resulting pellet was resuspended in fertilization medium and after appropriate dilution, 50 μl of this sperm suspension were added to 50 μl of the medium that contained the oocytes. The spermatozoa:oocyte ratio was 1000:1 (5×10^5 spermatozoa/mL). Gametes were coincubated at 39 °C in a humidified atmosphere of 5% CO_2 in air.

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2.4. In vitro culture

At 6 h after insemination, presumptive zygotes were removed from fertilization medium and washed three times in preequilibrated embryo culture medium. Subsequently, presumptive zygotes were transferred to a 4-well multidish (50 zygotes per well), each well containing 500 μl of the same medium under mineral oil, and cultured at 39 °C in 5% CO_2 in air for 12–15 h to assess fertilization parameters.

2.5. Assessment of sperm penetration

After culture, presumptive zygotes were mounted on slides and fixed in a solution of acetic acid:ethanol (1:3) for 48–72 h at room temperature. Oocytes were then stained with 1% lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope (magnification ×400). Oocytes with chromosomes at metaphase-II and an extruded polar body were considered matured. Oocytes were considered penetrated when they had one or more swollen sperm heads and/or male pronuclei, with their corresponding sperm tails present, and two polar bodies. Degenerated oocytes, immature oocytes, and oocytes with a broken oolemma or abnormal appearance of cytoplasm were not counted.

The fertilization parameters evaluated were: penetration (number of oocytes penetrated/total inseminated), monospermy (number of oocytes containing only one sperm head or one male pronuclei/total penetrated), number of spermatozoa/oocyte (mean number of spermatozoa in penetrated oocytes), and efficiency of fertilization (number of monospermic oocytes/total inseminated).

2.6. Experimental design

In an attempt to evaluate the effect of ultrashort periods of gamete co-incubation during pig *in vitro* fertilization, a total of 2833 *in vitro* matured oocytes from three replicates were inseminated with thawed spermatozoa and coincubated for 0.25, 1, 2, 3, 7, 10 min, and 6 h. The shorter co-incubation time, 0.25 min (15 s) was confirmed by the time we need to inseminate the oocytes in the microdrop and transfer them immediately to other drops with fresh IVF medium (without spermatozoa). Oocytes coincubated from 0.25 to 10 min were washed three times in fresh fertilization medium using a small bore pipette (approximately 150 μm of diameter) to eliminate spermatozoa not attached to the ZP and further cultured in fertilization medium (containing no spermatozoa) until the 6 h of co-incubation time were completed. After each period of co-incubation, 45–50 oocytes from each group were washed in DPBS and mounted on slide in 4 μl of a dilution of glycerol–DPBS (3:1) containing 10 μg/mL of Hoechst (Hoechst-33342). The number of spermatozoa bound to the zona was counted by using a fluorescence microscope (magnification ×400). After 6 h, the remaining oocytes from each co-incubation time were cultured in NCSU-23 medium for 12–15 h to assess fertilization parameters.

2.7. Statistical analysis

All data editing and statistical analysis were performed by SPSS, version 11.5 (SPSS Inc., Chicago, III). Data from three replicates were analyzed by ANOVA using the MIXED procedure according to a statistical model including the fixed effect of co-incubation time and the random effect of replicate. Penetration and monospermic rates, and efficiency data were mod-

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eled according to the binomial model of parameters as described by Fisz (Fisz, 1980) before analysis. When ANOVA showed a significant effect, values were compared using the Bonferroni test. The threshold for significance was set at $p < 0.05$. Results are expressed as least squares mean ± SEM.

3. Results

Co-incubation time significantly affected ($p < 0.001$) the penetration rate and the efficiency of fertilization (Fig. 1). When co-incubation times from 2 min to 6 h were used, the penetration rate was significantly ($p < 0.05$) higher (range: 53.5 ± 2.8–61.3 ± 2.6%) than that of 0.25 and 1 min groups (range: 27.4 ± 2.5–40.6 ± 2.9%). Similarly, the efficiency of fertilization reached a maximum for the 2 min of co-incubation group with values ranging between 32.3 ± 2.4 and 41.9 ± 2.5%. The reduction of co-incubation time did not affect the rate of monospermy, with values between 71.3 ± 3.4 and 80.2 ± 3.8%. The mean number of spermatozoa in penetrated oocytes followed a similar pattern to monospermy rate (range: 1.2 ± 0.4–1.4 ± 0.5).

The number of spermatozoa bound to the ZP (Fig. 2) increased significantly ($p < 0.02$) as the co-incubation time was increased (8.0 ± 2.8, 10.8 ± 2.8, 13.2 ± 2.8, 20.9 ± 2.8, 31.6 ± 2.8, 32.3 ± 2.8, and 44.2 ± 2.8, for 0.25, 1, 2, 3, 7, 10 min and 6 h, respectively).

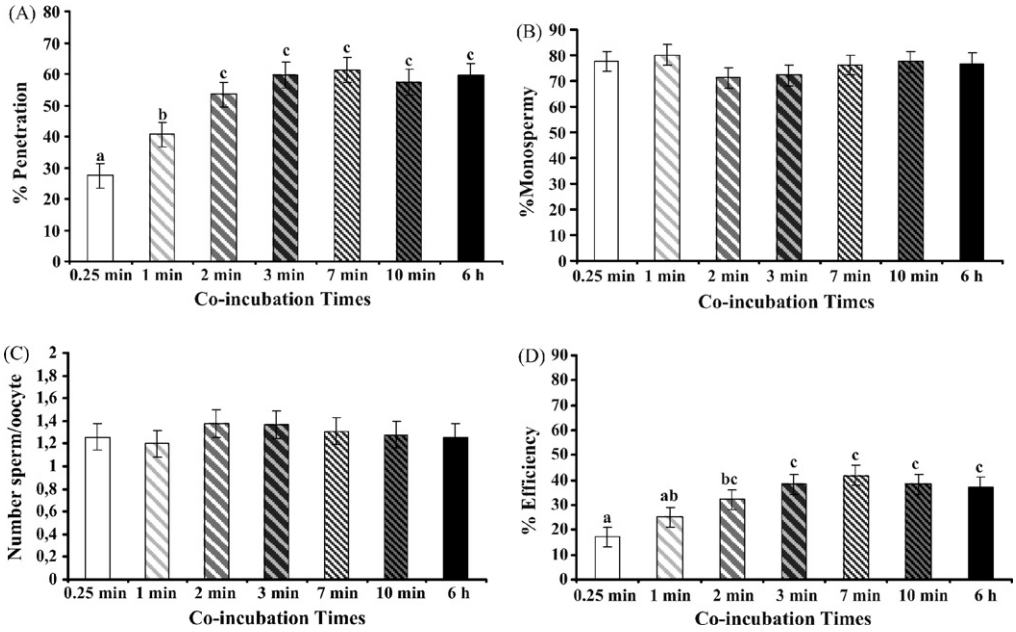

Fig. 1. Effect of short periods of spermatozoa–oocyte co-incubation during IVF on penetration rate (A), monospermy rate (B), number of sperm/oocyte (C), and efficiency of the IVF (D). Oocytes were coincubated with spermatozoa for 0.25 min ($n = 383$), 1 min ($n = 352$), 2 min ($n = 384$), 3 min ($n = 374$), 7 min ($n = 389$), 10 min ($n = 378$), and 6 h ($n = 258$). Oocytes coincubated from 0.25 to 10 min were washed three times in fresh fertilization medium using a small bore pipette to eliminate spermatozoa not attached to the zona pellucida and further cultured in fertilization medium (containing no spermatozoa) until the 6 h were completed. After insemination, presumptive zygotes were washed three times in embryo culture medium and cultured for 12–15 h to assess fertilization parameters a, b, c ($p < 0.03$, at least.).

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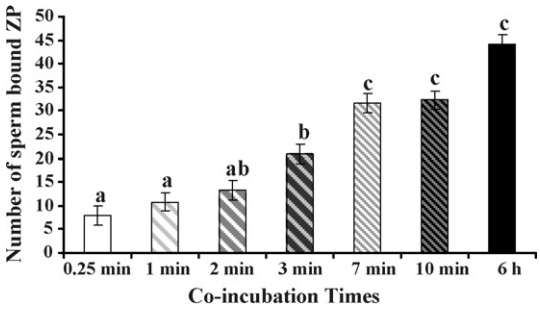

Fig. 2. Number of spermatozoa bound to the ZP. Oocytes were coincubated with spermatozoa for 0.25, 1, 2, 3, 7, 10 min and 6 h. Oocytes from each group of co-incubation (from 0.25 min to 6 h) were washed using a small bore pipette to eliminate spermatozoa not attached to the zona pellucida, transferred to fresh fertilization (containing no spermatozoa) and stained with Hoechst- 33342. The number of spermatozoa bound to the ZP was counted by using a fluorescence microscope at 400× magnification a, b, c, d ($p < 0.03$, at least).

4. Discussion

It is known that the oviduct microenvironment contributes to the completion of oocyte maturation in order to achieve an effective block to polyspermy, (Rodriguez-Martinez et al., 2005) and that under physiological conditions, monospermic penetration occurs in over 95% of the porcine oocytes (Hunter, 1990; Hunter, 1991; Wang et al., 1998). Contrarily to *in vivo* conditions, a high incidence of polyspermy occurs following *in vitro* fertilization of immature (Matas et al., 1996), *in vitro* matured (reviewed Abeydeera, 2002 and Funahashi, 2003) and *in vivo* matured (Coy et al., 1993a,b) oocytes, indicating suboptimal *in vitro* conditions and insufficient recognition, and interactions between both gametes. In an attempt to reduce *in vitro* polyspermic penetration of oocytes, many modifications have been made to porcine IVF protocols, including the manipulation of gamete co-incubation time (Cheng et al., 1986; Coy et al., 1993a; Martinez et al., 1996; Abeydeera and Day, 1997; Grupen and Nottle, 2000; Funahashi and Romar, 2004; Gil et al., 2004; Almiñana et al., 2005; Gil et al., 2007).

A reduction in co-incubation from the traditional 5–6 h to times as short as 10 min has been reported to improve penetration and blastocyst rates (Grupen and Nottle, 2000). However, a surprising increase in polyspermy and no significant differences in cleavage and blastocyst formation rates were found when this brief co-incubation time was used in our laboratory (Gil et al., 2004). Moreover, in the present study no significant differences were observed in penetration, monospermic and efficiency rates between 10 min and 6 h of co-incubation times. These contradictory results could be explained by the different conditions of IVF (Gil et al., 2007) or by differences among boars used (Almiñana et al., 2005). In this sense, it is widely accepted that penetration and polyspermy rates in pig IVF are affected by large variations among individual males. However, it has been demonstrated that all males do not respond to IVF conditions equally and that preliminary studies for each individual male are required in order to identify the optimal conditions for IVF and to decrease the variability among boars (Almiñana et al., 2005). In the present study we performed a preliminary screening to test the best IVF conditions for the boar used and, therefore, our results might be valid for spermatozoa from other boars subjected to similar screening for optimal IVF conditions.

Data from several previous publications on porcine IVF have demonstrated that there are enough spermatozoa bound to the ZP within 10 min of gamete co-incubation to fertilize a high

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number of oocytes (Grupen and Nottle, 2000; Gil et al., 2004; Almiñana et al., 2005; Gil et al., 2007) and that these bound spermatozoa require a maximum of 2 h to penetrate oocytes (Gil et al., 2007). Moreover, good penetration rates have been reported in human IVF using only 30 s of co-incubation (Bungum et al., 2006). All these findings encouraged us to investigate whether ultrashort gamete co-incubation times would be adequate to achieve good penetration rates and to reduce the incidence of polyspermic fertilization. For this reason, we reduced the co-incubation time from 6 h to 10, 7, 3, 2, 1, and 0.25 min. To the best of our knowledge this is the first study demonstrating successful fertilization with an ultrashort period of co-incubation in pigs.

Two further important conclusions may be drawn from the present study. First, it seems evident that the spermatozoa were able to interact immediately with the oocytes although the number of spermatozoa bound to the ZP increased significantly as the co-incubation time was increased. Second, a high penetration rate was obtained with co-incubation times as short as 2 min, without further increase among the longer co-incubation times. It is known that spermatozoa only bind to the ZP if they have previously undergone a series of biochemical transformations collectively called capacitation. This binding stimulates the spermatozoon to undergo the acrosome reaction, which allows penetration of the ZP and fertilization of the oocyte (reviewed in Yanagimachi, 1994). Because penetration and polyspermic fertilization occurs as early as 2–3 h postinsemination when frozen–thawed semen is used (Abeydeera and Day, 1997; Marchal et al., 2002; Gil et al., 2007) it could be assumed that a subpopulation of spermatozoa is "capacitated-like" at the moment of insemination. However, it has been demonstrated that fresh undiluted spermatozoa from untreated sperm-rich ejaculate fractions start to penetrate oocytes as early as 3 h after insemination and that capacitation treatments do not accelerate this process (Martinez et al., 1996). Therefore, it could be hypothesized that environmental conditions of co-culture are sufficient to induce a very fast process of capacitation and to ensure that there is a subpopulation of spermatozoa able to interact immediately with the oocytes and to bind to the ZP. This hypothesis is supported by data from the present study, which demonstrate that a sufficient number of spermatozoa are able to bind to the ZP within the first 2 min of co-incubation and to fertilize a high proportion of oocytes, similar to that obtained after 6 h of co-incubation time.

The high incidence of polyspermy in pigs leads to the hypothesis that maturation of the ooplasm and oocyte granules is insufficient to create the so called "zona block", even if the nuclear maturation is successful (Prather and Day, 1998). In the present experiment, while the number of spermatozoa bound to the ZP increased significantly as the co-incubation time was increased, the monospermic rates and the number of spermatozoa per oocyte penetrated were similar from 0.25 min to 6h of co-incubation. These results support the hypothesis that polyspermic penetration seems to be due to a delayed cortical reaction or an incomplete exocytosis of the cortical granules (CGs) on sperm penetration. *In vitro* matured and *in vivo* matured pig oocytes possess equal ability to release CGs on sperm penetration when they were co-cultured *in vitro*. Most of CGs from both groups of oocytes were released 6 h after IVF, regardless of whether they were polyspermic or monospermic oocytes (Wang et al., 1998). It is clear that such a release is too late in the light of our results and those previous studies on the time of spermatozoa-ZP interaction and penetration of spermatozoa (Grupen and Nottle, 2000; Gil et al., 2004; Almiñana et al., 2005; Gil et al., 2007). In agreement with Wang et al. (Wang et al., 1998) unknown changes in the extracellular matrix and/or cytoplasm of the oocytes while in the oviduct may play an important role(s) in the establishment of a functional block to polyspermy in pig oocytes.

In conclusion, this is the first study to demonstrate that a co-incubation time as brief as 2 min is long enough to obtain good fertilization rates similar to those achieved from current long-term exposure times (6 h) in porcine IVF. However, the reduction of co-incubation time from 6 h to

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0.25 min did not affect the monospermy or the number of spermatozoa in the oocytes. Further research in porcine IVF is required to achieve optimal final maturation of IVM oocytes for an effective block of polyspermy.

Acknowledgements

The authors wish to thank Professor W.M.C. Maxwell for helpful discussion and critical reading of the manuscript. The SENECA foundation of Murcia is acknowledged for funding a grant for C. Almiñana (00197/BPS/04). This study was supported by CICYT-FEDER (AGL2004-07546) and CDTI (04-0231) projects.

References

Abeydeera, L.R., 2002. *In vitro* production of embryos in swine. Theriogenology 57, 257–273.

Abeydeera, L.R., Day, B.N., 1997. Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified Tris-buffered medium with frozen–thawed ejaculated spermatozoa. Biol. Reprod. 57, 729–734.

Almiñana, C., Gil, M.A., Cuello, C., Roca, J., Vazquez, J.M., Rodriguez-Martinez, H., Martinez, E.A., 2005. Adjustments in IVF system for individual boars: value of additives and time of sperm–oocyte co-incubation. Theriogenology 64, 1783–1796.

Bungum, M., Bungum, L., Humaidan, P., 2006. A prospective study, using sibling oocytes, examining the effect of 30 s versus 90 min gamete co-incubation in IVF. Hum. Reprod. 21, 518–523.

Cheng, W.T.K., Polge, C., Moor, R.M., 1986. *In vitro* fertilization of pig and sheep oocytes. Theriogenology 25, 146.

Coy, P., Martinez, E., Ruiz, S., Vazquez, J.M., Roca, J., Matas, C., 1993a. *In vitro* fertilization of pig oocytes after different co-incubation intervals. Theriogenology 39, 1201–1208.

Coy, P., Martinez, E., Ruiz, S., Vazquez, J.M., Roca, J., Matas, C., 1993b. Sperm concentration influences fertilization and male pronuclear formation *in vitro* in pigs. Theriogenology 40, 539–546.

Day, B.N., 2000. Reproductive biotechnologies: current status in porcine reproduction. Anim. Reprod. Sci. 60–61, 161–172.

Fisz, M., 1980. Some probability distributions. In: De Krieger, R. (Ed.), Probability Theory and Mathematical Statistics. John Wiley and Sons, Inc., Malabar, FL, USA, pp. 129–174.

Funahashi, H., 2003. Polyspermic penetration in porcine IVM–IVF systems. Reprod. Fertil. Dev. 15, 167–177.

Funahashi, H., Day, B.N., 1997. Advances in *in vitro* production of pig embryos. J. Reprod. Fertil. 52, 271–283.

Funahashi, H., Romar, R., 2004. Reduction of the incidence of polyspermic penetration into porcine oocytes by pretreatment of fresh spermatozoa with adenosine and a transient co-incubation of the gametes with caffeine. Reproduction 128, 789–800.

Gil, M.A., Abeydeera, L.R., Day, B.N., Vazquez, J.M., Roca, J., Martinez, E.A., 2003. Effect of the volume of medium and number of oocytes during *in vitro* fertilization on embryo development in pigs. Theriogenology 60, 767–776.

Gil, M.A., Ruiz, M., Vazquez, J.M., Roca, J., Day, B.N., Martinez, E.A., 2004. Effect of short period of sperm–oocyte co-incubation during *in vitro* fertilization on embryo development in pigs. Theriogenology 62, 544–552.

Gil, M.A., Almiñana, C., Cuello, C., Parrilla, I., Roca, J., Vazquez, J.M., Martinez, E.A., 2007. Brief co-incubation of gametes in porcine *in vitro* fertilization: role of sperm: oocyte ratio and postco-incubation medium. Theriogenology 67, 620–626.

Grupen, C.G., Nottle, M.B., 2000. A simple modification of the *in vitro* fertilization procedure. Theriogenology 53, 422.

Hunter, R.H.F., 1990. Fertilization in pig eggs *in vivo* and *in vitro*. J. Reprod. Fertil. Suppl. 40, 211–226.

Hunter, R.H.F., 1991. Oviduct function in pigs, with particular reference to the pathological condition of polyspermy. Mol. Reprod. Dev. 29, 385–391.

Marchal, R., Pelaez, J., Terqui, M., Mermillod, P., 2002. Effect of sperm survival and CTC staining pattern on *in vitro* fertilization of porcine oocytes. Theriogenology 57, 1917–1927.

Martinez, E.A., Vazquez, J.M., Matas, C., Gadea, J., Alonso, M.I., Roca, J., 1996. Oocyte penetration by fresh or stored diluted boar spermatozoa before and after *in vitro* capacitation treatments. Biol. Reprod. 55, 134–140.

Matas, C., Martinez, E., Vazquez, J.M., Roca, J., Gadea, J., 1996. *In vitro* penetration assay of boar sperm fertility: Effect of various factors on the penetrability of immature pig oocytes. Theriogenology 46, 503–513.

Please cite this article in press as: Almiñana, C. et al., Effects of ultrashort gamete co-incubation time on porcine *in vitro* fertilization, Anim. Reprod. Sci. (2007), doi:10.1016/j.anireprosci.2007.05.017

Mattioli, M., Bacci, M.L., Galeati, G., Seren, E., 1989. Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 31, 1201–1207.

Niwa, K., 1993. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs. J. Reprod. Fertil. Suppl. 48, 49–59.

Peters, R.M., Wells, K.D., 1993. Culture of pig embryos. J. Reprod. Fertil. 48, 61–73.

Prather, R.S., Day, B.N., 1998. Practical considerations for the *in vitro* productions of pig embrions. Theriogenology 49, 23–32.

Pursel, V.G., Johnson, L.A., 1974. Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation. Theriogenology. 1 (2), 63–68.

Roca, J., Carvajal, G., Lucas, X., Vazquez, J.M., Martinez, E.A., 2003. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen–thawed spermatozoa. Theriogenology 60, 77–87.

Rodriguez-Martinez, H., Saravia, F., Wallgren, M., Tienthai, P., Johannisson, A., Vazquez, J.M., Martinez, E., Roca, J., Sanz, L., Calvete, J.J., 2005. Boar spermatozoa in the oviduct. Theriogenology 63, 514–535.

Suzuki, K., Eriksson, B., Shimizu, H., Nagai, T., Rodríguez-Martinez, H., 2000. Effect of hyaluronanon monospermic penetration of porcine oocytes fertilizated *in vitro*. Int. J. Androl. 23, 13–21.

Vazquez, J.M., Martinez, E., Roca, J., Coy, P., Pastor, L.M., 1993. Acrosome reaction of boar spermatozoa in homologous *in vitro* fertilization. Mol. Reprod. Dev. 36, 84–88.

Wang, W.H., Abeydeera, L.R., Prather, R.S., Day, B.N., 1998. Morphologic comparison of ovulated and *in vitro*-matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. Mol. Reprod. Dev. 49, 308–316.

Yanagimachi, R., 1994. Fertility of mammalian spermatozoa: its development and relativity. Zygote 2, 371–372.

Please cite this article in press as: Almiñana, C. et al., Effects of ultrashort gamete co-incubation time on porcine *in vitro* fertilization, Anim. Reprod. Sci. (2007), doi:10.1016/j.anireprosci.2007.05.017

Artículo 4

Reprod Dom Anim; doi: 10.1111/j.1439-0531.2007.00995.x
ISSN 0936-6768

In Vitro Fertilization (IVF) in Straws and a Short Gamete Coincubation Time Improves the Efficiency of Porcine IVF

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Contents

The present study was designed to evaluate three different *in vitro* fertilization (IVF) systems: a straw-IVF system with 10 min of coincubation, a straw-IVF system with 6-h coincubation and the microdrop-IVF system with 6-h coincubation (the traditional IVF system used routinely in most of IVF laboratories) in an attempt to reduce polyspermic penetration (Experiment 1). When the straw-IVF system was tested in combination with two coincubation times, the use of 10 min of coincubation significantly increased ($p < 0.001$) the penetration rate and the efficiency of fertilization (67.7 ± 6.4% vs 31.9 ± 6.5% and 41.5 ± 2.5% vs 17.6 ± 2.5% for 10 min and 6 h, respectively), while there were no significant differences in the incidence of monospermy between both systems (64.3 ± 5.1% and 67.7 ± 3.4%, for 10 min and 6 h, respectively). The penetration rate in the 6-h microdrop-IVF system was higher (93.8 ± 3.6%; $p < 0.001$) compared with the 10-min straw-IVF system (67.7 ± 6.4%), however, monospermy was severely reduced (25.0 ± 4.3% vs 67.7 ± 3.4%, for the 6-h microdrop-IVF system and 10-min straw-IVF system, respectively). The efficiency of the IVF showed similar values between microdrop and 6-h straw-IVF systems, but efficiency was significantly improved ($p < 0.05$) when the 10-min straw-IVF system was used. Experiment 2 was designed to compare porcine *in vitro* embryo production in two IVF systems, the 6-h microdrop-IVF system (1000 sperm per oocyte) and 10-min straw-IVF system (30 000 sperm per oocyte). The blastocyst formation rates tended ($p = 0.06$) to be higher when the 10-min straw-IVF system was used compared with the 6-h microdrop-IVF system. In addition, the number of total cells per blastocyst increased significantly ($p < 0.05$) in the 10-min straw-IVF system. These results showed that the 10-min straw-IVF system is an effective way to decrease polyspermic penetration, and improve the efficiency of fertilization and the quality of blastocysts in terms of cell number per embryo.

Introduction

In vitro production of mammalian embryos is no longer an exclusive technique for research laboratories. It is considered as an initial step to obtain a large source of embryos from abattoir-derived ovaries for the application of new biotechnologies. In pigs, the efficiency of embryos produced *in vitro* is remarkably low because of the unacceptable high incidence of polyspermy obtained during *in vitro* fertilization (IVF) (Niwa 1993; Matas et al. 1996; Abeydeera and Day 1997; Funahashi and Day 1997; Wang et al. 1997; Abeydeera 2002), and the poor quality of blastocysts derived following *in vitro* culture of putative zygotes (Han et al. 1999a,b; McCauley et al. 2003). For this reason, the improvement of IVF protocols is critical to overcome the low rates of porcine blastocysts currently produced *in vitro*.

The conditions under which IVF is performed differ among laboratories. However, a common factor in current IVF systems is the exposition of oocytes to an excessive and non-physiological number of sperm during gamete coincubation. This high number of spermatozoa per oocyte causes simultaneous sperm penetration and results in polyspermic fertilization (Wang et al. 2003). In addition, the environment conditions during *in vitro* gamete coincubation are far from the oviductal environment in the female genital tract. These IVF conditions appear to override the *in vivo* strategies for sperm capacitation and natural selection of spermatozoa after insemination (Hunter and Rodriguez-Martinez 2004). Thus, a reduction in spermatozoa number during IVF has been studied in order to decrease polyspermic penetration, however, it also reduces overall sperm penetration rates (Abeydeera and Day 1997) resulting in low efficiency of fertilization. New strategies such as the straw-IVF system (Li et al. 2003), the climbing-over-wall method (Funahashi and Nagai 2000), or microfluidic technology (Beebe et al. 2002; Clark et al. 2002, 2003, 2005) have been reported in an attempt to mimic the process of *in vivo* fertilization in the oviductal tract, where spermatozoa are gradually capacitated during transport to the site of fertilization, (Rodriguez-Martinez et al. 2005), reducing the incidence of polyspermic fertilization (Funahashi and Nagai 2000).

On the other hand, several studies suggest that longer gamete coincubation times (Coy et al. 1993a; Martinez et al. 1996; Abeydeera and Day 1997; Marchal et al. 2002; Gil et al. 2007) parallel higher penetration rates and higher incidence of polyspermic penetration. In addition, it has been reported that there are sufficient spermatozoa bound to zona pellucida within the first 2–10 min of gamete coincubation to penetrate a high number of oocytes (Gil et al. 2004a, 2007; Almiñana et al. 2007) and, therefore, a short gamete coincubation time (10 min) has been suggested as an alternative method to increase the efficiency of IVF of porcine oocytes (Grupen and Nottle 2000; Funahashi and Romar 2004; Gil et al. 2004a; Almiñana et al. 2005).

Based on these findings, the present study was designed to evaluate whether the combination of a straw-IVF system and a short coincubation time (10 min) is a suitable strategy to decrease polyspermic fertilization and improve *in vitro* production of porcine blastocysts.

Materials and Methods

Culture media

The medium used for the collection and washing of cumulus–oocyte complexes (COCs) was Dulbecco's phosphate-buffered saline (DPBS) medium composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4

and 1.46 mM $CaCl_2.2H_2O$ supplemented with 4 mg/ml bovine serum albumin [BSA; (fraction V)], 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 μg/ml Kanamycin (mDPBS). The oocyte maturation medium was BSA-free North Carolina State University (NCSU)-23 (Peters and Wells 1993) supplemented with 10% (v : v) porcine follicular fluid, 0.1 mg/ml cysteine and 10 ng/ml epidermal growth factor. The fertilization medium used was modified Brackett and Oliphant (mBO) medium (Brackett and Oliphant 1975) supplemented with 2 mM caffeine and 0.2% BSA, but without casein phosphopeptides. The embryo culture medium was NCSU-23 supplemented with 0.4% BSA.

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain).

Recovery and *in vitro* maturation of cumulus–oocyte complexes

Ovaries were obtained from prepuberal gilts at a local slaughterhouse and transported to the laboratory at 35°C within 1 h after collection in 0.9% NaCl containing 70 μg/ml Kanamycin. After the ovaries were washed three times in NaCl solution, follicles 3–6 mm in diameter were aspirated using an 18-gauge needle connected to a 10-ml disposable syringe. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times in maturation medium and matured in a 4-well multidish (Nunc, Roskilde, Denmark) containing 150–200 COCs per well in 500 μl of maturation medium supplemented with 10 IU/ml eCG (Foligon; Intervet International B.V., Boxmeer, the Netherlands) and 10 IU/ml hCG (Veterin Corion; Divasa Farmavic, S.A., Barcelona, Spain) for 20–22 h and then for another 20–22 h in maturation medium without hormones. Oocyte maturation was carried out under mineral oil at 39°C in an atmosphere of 5% CO_2 in air.

In vitro fertilization

Fertilization was performed in two different systems according to the experimental design. After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium by vortexing for 2 min, 1660 rounds/min. Oocytes were washed twice in maturation medium and three times in pre-equilibrated fertilization medium. For the microdrop-IVF system, 30 denuded oocytes were placed in 50-μl drops of IVF medium in a 35 × 10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) under mineral oil and held at 39°C in an atmosphere of 5% CO_2 in air for about 30 min until addition of spermatozoa. For the straw-IVF system, denuded oocytes were placed in 150-μl drops of IVF medium in a 35 × 10 mm Petri dish under the same conditions as the microdrop-IVF system until oocytes and spermatozoa were loaded in straws.

For IVF, 100 μl of thawed semen were washed three times by centrifugation at 1900 × ***g*** for 3 min in mDPBS. The resulting pellet was resuspended in fertilization medium and diluted according to the experimental design. For the microdrop-IVF system, 50 μl of this sperm suspension was added to a 50-μl drop of fertilization medium containing the oocytes. The straw-IVF system was performed as described by Li et al. (2003) with a few modifications. Briefly, before loading the straws, the plugs of the straws were cut and connected to a 1-ml syringe through a rubber connector. The straws were loaded in the following sequence: 25 μl of fertilization medium that contained 30 denuded oocytes, 100 μl of fertilization medium (containing no spermatozoa) and 25 μl of the sperm suspension. Then, the rubber connector was carefully eliminated and straws were placed horizontally in plates in the incubator. Gametes were coincubated at 39°C in humidified atmosphere of 5% CO_2 and air according to the experimental design.

In vitro culture

Presumptive zygotes from both systems were removed from fertilization medium and washed three times in pre-equilibrated embryo culture medium. Subsequently, presumptive zygotes were transferred to a 4-well multidish (50 zygotes per well), each well containing 500 μl of the same medium under mineral oil, and cultured at 39°C, 5% CO_2 in air for 16 h to assess fertilization parameters or for 168 h to assess embryo development.

Assessment of sperm penetration and embryo development

To evaluate fertilization parameters, presumptive zygotes were mounted on slides, fixed in a solution of acetic acid : ethanol (1 : 3) for 48–72 h at room temperature, stained with 1% lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of 400×. Oocytes were considered penetrated when they had one or more swollen sperm heads and/or male pronuclei, with their corresponding sperm tails present, and two polar bodies. Degenerated oocytes, immature oocytes and oocytes with a broken oolemma or abnormal appearance of cytoplasm were not counted. The fertilization parameters evaluated were: penetration (number of oocytes penetrated/total inseminated), monospermy (number of oocytes containing only one male pronucleus/total penetrated), number of spermatozoa/oocyte (number of spermatozoa in penetrated oocytes) and efficiency of fertilization (number of monospermic oocytes/total inseminated).

To examine the ability of embryos to develop to the blastocyst stage *in vitro*, presumptive zygotes were cultured for 168 h and the blastocyst formation (number of blastocyst/total cultivated) was evaluated under a stereomicroscope. An embryo with a clear blastocele was defined as a blastocyst. The total cell number, as an indicator of embryo quality, was evaluated by mounting each blastocyst on slides in 4 μl of a dilution of Glycerol–DPBS (3 : 1) containing 10 μg/ml of Hoechst-33342 followed by examination using fluorescent microscopy.

Experimental design

Experiment 1. Effect of the straw-IVF system with 10-min coincubation (10-min straw-IVF system) on IVF parameters

In this experiment, the combined effect of a straw-IVF system and a short period of gamete coincubation

(10 min) was compared with straw-IVF system with 6 h of coincubation (6-h straw-IVF system) and to the microdrop-IVF system (6 h of coincubation) in an attempt to reduce polyspermic penetration. Various sperm : oocyte ratios (20 000, 30 000, 40 000 and 50 000) were used for the 10-min and 6-h straw-IVF systems. These ratios were selected from previous experiments (data not shown). A ratio of 1000 spermatozoa per oocyte was used for the microdrop-IVF system. Oocytes from the 10-min straw-IVF system were washed three times in fertilization medium to remove spermatozoa not bound to the zona and transferred to clean microdrops of the same medium (containing no sperm) for 6 h. Six hours after insemination, oocytes from both IVF systems were washed three times in pre-equilibrated embryo culture medium and cultured for 16 h to assess fertilization parameters. A total of 723 *in vitro* matured oocytes, from three replicates, were used in this experiment.

Experiment 2. Effect of 10-min straw-IVF system on in vitro embryo development

This experiment was designed to compare porcine *in vitro* embryo production in two IVF systems, the 6-h microdrop-IVF system (1000 sperm per oocyte) and the 10-min straw-IVF system (30 000 sperm per oocyte), based on the results of Experiment 1. Presumptive zygotes from both systems were cultured in NCSU-23 medium for 168 h to assess embryo development. A total of 471 *in vitro* matured oocytes from three replicates were used in this experiment.

Statistical analysis

All data editing and statistical analysis were performed by SPSS, version 11.5 (SPSS Inc., Chicago, IL, USA). Data from three replicates were analysed by analysis of variance using the mixed procedure. Penetration and monospermic rates, efficiency data and blastocyst formation were modelled according to the binomial model of parameters as described by Fisz (1980) before analysis. When analysis of variance showed a significant effect, values were compared using the Bonferroni test. The threshold for significance was set at $p < 0.05$. Results are expressed as least squares mean ± SEM.

Results

Experiment 1. Effect of the 10-min straw-IVF system on IVF parameters

As shown in Table 1, when the straw-IVF system was tested in combination with two coincubation times, the use of 10 min of sperm–oocyte coincubation significantly increased ($p < 0.001$) the penetration rate and efficiency of fertilization (67.7 ± 6.4% vs 31.19 ± 6.5% and 41.5 ± 2.5% vs 17.6 ± 2.5% for 10 min and 6 h, respectively), while there were no significant differences in the incidence of monospermy between either system (64.3 ± 5.1% and 67.7 ± 3.4%, for 10 min and 6 h, respectively). Regarding the different sperm : oocyte ratios tested in the straw-IVF systems, no significant differences were observed for any of the IVF parameters evaluated within of each system. However, the higher sperm : oocyte ratio (50 000) showed a tendency to increase the penetration rate ($p = 0.10$) and the mean number of spermatozoa/oocyte penetrated ($p = 0.09$), tending to decrease the monospermy rate ($p = 0.12$). When the microdrop-IVF system was compared with straw-IVF systems, the penetration rate was significantly higher (95.5 ± 5.6%; $p < 0.001$) and monospermy rate was lower ($p < 0.001$) achieving values as low as 25.0 ± 4.3%. The efficiency of IVF showed similar values between microdrop and 6-h straw-IVF systems, but it was significantly higher ($p < 0.05$) when the 10-min straw-IVF system was used.

Experiment 2. Effect of 10-min straw-IVF system on *in vitro* embryo development

The blastocyst formation rates tended ($p = 0.06$) to be higher when the 10-min straw-IVF system was used in comparison with microdrop-IVF system. In addition, the number of total cells per blastocyst increased significantly ($p < 0.05$) in the straw-IVF system (Fig. 1).

Table 1. Effect of two *in vitro* fertilization (IVF) systems, microdrop and straw, in combination with two gamete coincubation times, 6 h and 10 min, on IVF parameters

IVF system	Coincubation time	Sperm : oocyte ratio	No. oocytes	Penetration (%)	Monospermy (%)	EO	Efficiency (%)
Microdrop	6 h	1000	144	93.8 ± 3.6 ^{d}	25.0 ± 4.3 ^{b}	2.90 ± 0.10 ^{b}	18.8 ± 3.5 ^{a}
Straw- IVF system	6 h	20 000	69	26.1 ± 5.2 ^{b}	77.8 ± 10.9^{a}	1.33 ± 0.29^{ac}	17.3 ± 4.8 ^{a}
		30 000	72	25.0 ± 5.1 ^{b}	72.2 ± 10.9^{a}	1.72 ± 0.29 ^{ac}	16.0 ± 4.8^{a}
		40 000	66	34.8 ± 5.3 ^{ab}	65.2 ± 9.6^{a}	1.56 ± 0.25 ^{ac}	20.3 ± 5.0 ^{ac}
		50 000	75	41.3 ± 5.0 ^{ab}	41.9 ± 8.3^{ab}	2.29 ± 0.22 ^{ab}	16.9 ± 4.9 ^{a}
Straw- IVF system	10 min	20 000	69	58.0 ± 5.2 ^{ac}	77.5 ± 7.3 ^{a}	1.32 ± 0.19 ^{c}	41.3 ± 5.0 ^{bc}
		30 000	71	70.4 ± 5.1 ^{c}	74.0 ± 6.5 ^{a}	1.28 ± 0.17 ^{c}	46.3 ± 4.8 ^{b}
		40 000	74	68.9 ± 5.0 ^{c}	60.8 ± 6.5 ^{a}	1.56 ± 0.17 ^{ac}	37.3 ± 4.8 ^{abc}
		50 000	83	72.3 ± 4.7 ^{c}	58.3 ± 6.0^{a}	1.65 ± 0.15 ^{ac}	41.2 ± 4.7 ^{bc}

Different superscripts letter within the same column represent a significant difference ($p < 0.05$) at least.
Penetration: number of oocytes penetrated/total inseminated.
Monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated.
EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes.
Efficiency: number of monospermic oocytes/total inseminated.

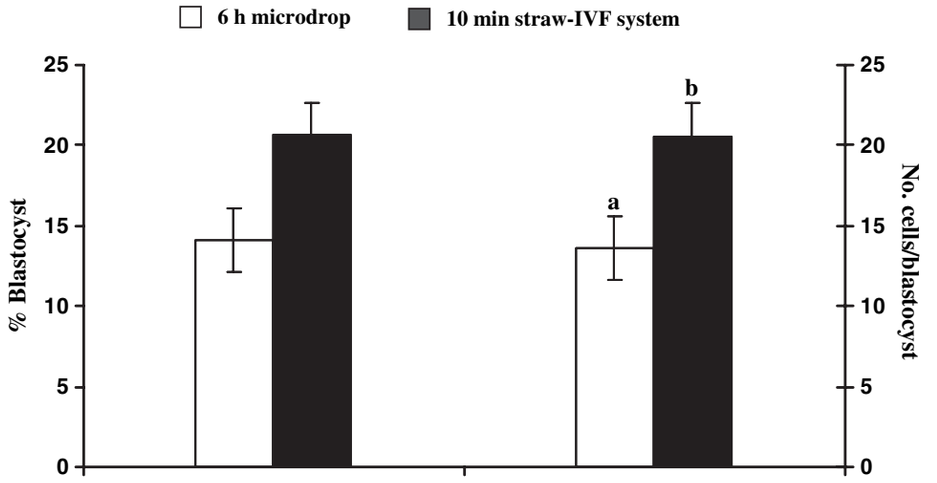

Fig. 1. Effect of a 10-min straw-*in vitro* fertilization (IVF) system during IVF on *in vitro* embryo development. Oocytes from three replicates were fertilized with frozen-thawed spermatozoa using a microdrop-IVF system (1000 sperm per oocyte and 6 h of coincubation time) and using the straw-IVF system (30 000 sperm per oocyte and 10 min of coincubation). Oocytes from the 10-min group were washed three times by mechanical pipetting in modified Brackett and Oliphant medium to remove spermatozoa not bound to the zona and transferred to a microdrop containing the same medium (containing no sperm) for 6 h. After 6 h, presumptive zygotes from both groups were cultured in NCSU-23 medium for 168 h to assess embryo development. $^{a,b}(p < 0.05)$ (% blastocysts, percentage of blastocysts/total oocytes inseminated; No. Cells/blastocyst, total number of cells/blastocyst)

Discussion

Low efficiency of porcine IVF, related mainly to the high incidence of polyspermic penetration, has prompted the development of new IVF methodologies. Some of these new IVF strategies include changes in both the IVF system (Funahashi and Nagai 2000; Beebe et al. 2002; Li et al. 2003; Clark et al. 2005) and gamete coincubation time (Funahashi and Romar 2004; Gil et al. 2004a, 2007; Almiñana et al. 2005). These strategies have concentrated on reducing the exposure of oocytes to an excessive number of spermatozoa by limiting both the large number of spermatozoa that reach the oocyte surface and the gamete coincubation time. Knowing full well that the microdrop-IVF system used in the present study leads to low monospermy rates, we attempted to use an alternative IVF system (straw-IVF system) that could allow the use of a wide range of sperm : oocyte ratios, and a brief gamete coincubation (10 min). Results demonstrated that the combination of both strategies is an effective way to decrease polyspermic penetration and improve efficiency of IVF compared with the microdrop-IVF system.

The higher incidence of monospermy achieved with the straw-IVF system compared with the microdrop-IVF system ($p < 0.001$) suggests that this methodology allows a gradual exposure of oocytes to a suitable number of capacitated spermatozoa, thus avoiding the massive presence of capacitated spermatozoa in the immediate vicinity of oocytes as occurs with use of the microdrop-IVF system. As suggested by Li et al. (2003), the use of this straw-IVF system appears to allow very motile spermatozoa to swim towards and fertilize oocytes, while inducing the acrosome reaction in spermatozoa at different times. As a result, the simultaneous sperm penetration, which seems to be one of the main reasons for polyspermy of porcine oocytes (Wang et al. 2003), is reduced.

Moreover, in the present study, different sperm : oocyte ratios were tested through the straw-IVF system on IVF parameters. Although there was a great increase in the spermatozoa number among each sperm : oocyte ratio tested, from 20 000 to 50 000, no significant differences were observed in either coincubation time (6 h and 10 min) for any of the IVF parameters evaluated. These results differ from those previously reported, where the sperm : oocyte ratio highly influenced polyspermic penetration and efficiency of porcine IVF in the microdrop-IVF system (Rath 1992; Coy et al. 1993b; Xu et al. 1996; Gil et al. 2004b). The fact that no increase in penetration and polyspermy rates was observed when the sperm : oocyte ratio was increased from 20 000 to 50 000 sperm per oocyte in the straw-IVF system reinforces the hypothesis mentioned above suggesting that in this system only motile spermatozoa successfully reached the site of fertilization, avoiding a massive contact between spermatozoa and oocytes.

The influence of different sperm : oocyte ratios combined with brief coincubation time have been evaluated earlier in our laboratory using the microdrop-IVF system. Those results showed that longer coincubation times required lower sperm : oocyte (500 sperm : oocyte) ratios than short coincubation times (1000, 1500 or 2000 sperm : oocyte) in order to obtain the same penetration rate (Gil et al. 2007). However, the combination of these two strategies (different sperm : oocytes ratios and brief coincubation) with the straw-IVF system revealed that, independent of the sperm : oocyte ratio, coincubation times as long as 6 h significantly decrease the penetration rates and, as a consequence, the efficiency of IVF. A possible explanation for these unexpected low penetration rates may be that oocytes remaining inside the straw suffered oxidative damage. It is known that frozen-thawed spermatozoa release reactive oxygen species (ROS) as products of metabolism

and cell death that could interact in the events associated with fertilization (Kessopoulou et al. 1992; Mammoto et al. 1996). Thus the high amount of frozen-thawed spermatozoa present inside the straw could release enough ROS to affect fertilization. The transfer of the oocytes at 10 min of coincubation to a fresh drop would remove the excess dead sperm and benefit the fertilization. This hypothesis was proposed by Gil et al. (2004a) in a previous report, where oocytes obtained after 10 min of coincubation were pipetted and transferred to a fresh drop of IVF medium showed higher penetration rates with higher incidence of polyspermic penetration than those coincubated with spermatozoa for 6 h. Those results differ from our study, where no significant differences were observed in the incidence of monospermy between 6 h and 10 min of coincubation. These discrepancies could be attributed to the different IVF systems together with the different IVF conditions used.

Based on our results, it is clear that the straw-IVF system combined with a 10-min coincubation, resulted in higher monospermy rates than the microdrop-IVF system where the monospermy rate was as low as 25%. The significant reduction of polyspermy achieved by our system lead us to investigate whether the *in vitro* production of embryos could be improved by this method. In the present study, no significant differences were observed in the blastocyst formation rate between the microdrop-IVF system and 10-min straw-IVF system, in spite of the fact that the efficiency of fertilization was doubled when the 10-min straw-IVF system was used. Nevertheless the use of the 10-min straw-IVF system showed a tendency ($p = 0.06$) to increase blastocyst formation rate and increase ($p < 0.05$) the quality of blastocysts in terms of total cell number per embryo. These results support previous reports indicating that polyspermic *in vitro* maturation-IVF porcine embryos develop to the blastocyst stage *in vitro* at a similar rate to monospermic embryos (Han et al. 1999a,b; McCauley et al. 2003) and also suggests that a higher monospermy rate might enhance embryo quality.

In conclusion, our study demonstrates that a straw-IVF system in combination with a 10-min coincubation increased monospermic penetration, the efficiency of fertilization and the quality of blastocysts in terms of cell number/embryo as compared with the microdrop-IVF system. In addition, this new strategy allowed the use of a wide range of sperm : oocyte ratios.

Acknowledgements

The authors wish to thank Dr Tod McCauley for helpful discussion and critical reading of the manuscript. The SENECA foundation of Murcia is acknowledged for funding a grant for C. Almiñana (00197/BPS/04). This study was supported by CICYT-FEDER (AGL2004-07546) and CDTI (04-0231) projects.

References

Abeydeera LR, 2002: In vitro production of embryos in swine. Theriogenology **57,** 257–273.

Abeydeera LR, Day BN, 1997: Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol Reprod **57,** 729–734.

Almiñana C, Gil MA, Cuello C, Roca J, Vazquez JM, Rodriguez-Martinez H, Martinez EA, 2005: Adjustments in IVF system for individual boars: value of additives and time of sperm-oocyte co-incubation. Theriogenology **64,** 1783–1796.

Almiñana C, Gil MA, Cuello C, Parrilla I, Roca J, Vazquez JM, Martinez EA, 2007: Effects of ultrashort gamete coincubation time on porcine in vitro fertilization. Anim Reprod Sci. doi:10.1016/j.anireprosci.2007.05.017

Beebe DJ, Wheeler BM, Zeringue H, Walters E, Raty S, 2002: Microfluidic technology for assisted reproduction. Theriogenology **57,** 125–135.

Brackett BG, Oliphant G, 1975: Capacitation of rabbit spermatozoa in vitro. Biol Reprod **12,** 260–274.

Clark SG, Walters EM, Beebe DJ, Wheeler BM, 2002: In vitro fertilization of porcine oocytes in polydimethylsiloxane (PDMS)-glass microchannels. Biol Reprod Suppl **1,** 528.

Clark SG, Walters EM, Beebe DJ, Wheeler BM, 2003: A novel integrated in vitro maturation and in vitro fertilization system for swine. Theriogenology **59,** 441.

Clark SG, Haubert K, Beebe DJ, Ferguson E, Wheeler BM, 2005: Reduction of polyspermic penetration using biomimetic microfluidic technology during in vitro fertilization. Lab chip **5,** 1229–1232.

Coy P, Martinez E, Ruiz S, Vazquez JM, Roca J, Matas C et al., 1993a: In vitro fertilization of pig oocytes after different coincubation intervals. Theriogenology **39,** 1201–1208.

Coy P, Martinez E, Ruiz S, Vazquez JM, Roca J, Matas C, 1993b: Sperm concentration influences fertilization and male pronuclear formation in vitro in pigs. Theriogenology **40,** 539–546.

Fisz M, 1980: Some probability distributions. In: De Krieger R (ed.), Probability Theory and Mathematical Statistics. John Wiley & Sons, Inc., Malabar, FL, USA, pp. 129–174.

Funahashi H, Day BN, 1997: Advances in in vitro production of pig embryos. J Reprod Fertil **52,** 271–283.

Funahashi H, Nagai T, 2000: Sperm selection by a climbing-over-a-wall method reduces the incidence of polyspermic penetration of porcine oocytes. J Reprod Dev **46,** 319–324.

Funahashi H, Romar R, 2004: Reduction of the incidence of polyspermic penetration into porcine oocytes by pre-treatment of fresh spermatozoa with adenosine and a transient co-incubation of the gametes with caffeine. Reproduction **128,** 789–800.

Gil MA, Ruiz M, Vazquez JM, Roca J, Day BN, Martinez EA, 2004a: Effect of short period of sperm-oocyte coincubation during in vitro fertilization on embryo development in pigs. Theriogenology **62,** 544–552.

Gil MA, Ruiz M, Cuello C, Vazquez JM, Roca J, Martinez EA, 2004b: Influence of sperm:oocyte ratio during in vitro fertilization of in vitro matured cumulus-intact pig oocytes on fertilization parameters and embryo development. Theriogenology **61,** 551–560.

Gil MA, Almiñana C, Cuello C, Parrilla I, Roca J, Vazquez JM, Martinez EA, 2007: Brief coincubation of gametes in porcine in vitro fertilization: role of sperm:oocyte ratio and post-coincubation medium. Theriogenology **67,** 620–626.

Grupen CG, Nottle MB, 2000: A simple modification of the in vitro fertilization procedure. Theriogenology **53,** 422.

Han YM, Abeeydera LR, Kim JH, Moon HB, Cabot RA, Day BN, 1999a: Growth retardation of inner cell mass cells in polyspermic porcine embryos produced in vitro. Biol Reprod **60,** 1110–1113.

Han YM, Wang WH, Abeeydera LR, Petersen AL, Kim JH, Murphy C, 1999b: Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos. Biol Reprod **61,** 1340–1346.

Hunter RH, Rodriguez-Martinez H, 2004: Capacitation of mammalian spermatozoa in vivo, with a specific focus on events in the fallopian tubes. Mol Reprod Dev **67,** 243–250.

Kessopoulou E, Tomlinson MJ, Barratt CL, Bolton AE, Cooke ID, 1992: Origin of reactive oxygen species in human semen: spermatozoa or leucocytes? J Reprod Fertil **94,** 463–470.

Li YH, Ma W, Li M, Hou Y, Jiao LH, Wang WH, 2003: Reduced polyspermic penetration in porcine oocytes inseminated in a new in vitro fertilization (IVF) system: straw IVF. Biol Reprod **69,** 580–585.

Mammoto A, Masumoto N, Tahara M, Ikebuchi Y, Ohmichi M, Tasaka K, Miyake A, 1996: Reactive oxygen species block sperm-egg fusion via oxidation of sperm sulfhydryl proteins in mice. Biol Reprod **55,** 1063–1068.

Marchal R, Pelaez J, Terqui M, Mermillod P, 2002: Effect of sperm survival and CTC staining pattern on in vitro fertilization of porcine oocytes. Theriogenology **57,** 1917–1927.

Martinez EA, Vazquez JM, Matas C, Gadea J, Alonso MI, Roca J, 1996: Oocyte penetration by fresh or stored diluted boar spermatozoa before and after in vitro capacitation treatments. Biol Reprod **55,** 134–140.

Matas C, Martinez E, Vazquez JM, Roca J, Gadea J, 1996: In vitro penetration assay of boar sperm fertility: effect of various factors on the penetrability of immature pig oocytes. Theriogenology **46,** 503–513.

McCauley TC, Mazza MR, Didion BA, Mao J, Wu G, Coppola G, 2003: Chromosomal abnormalities in day-6, in vitro produced embryos. Theriogenology **60,** 1569–1580.

Niwa K, 1993: Effectiveness of in vitro maturation and in vitro fertilization techniques in pigs. J Reprod Fertil Suppl **48,** 49–59.

Peters RM, Wells KD, 1993: Culture of pig embryos. J Reprod Fertil **48,** 61–73.

Rath D, 1992: Experiments to improve in vitro fertilization techniques for in vivo matured porcine oocytes. Theriogenology **37,** 885–886.

Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vazquez JM, Martinez E, Roca J, Sanz L, Calvete JJ, 2005: Boar spermatozoa in the oviduct. Theriogenology **63,** 514–535.

Wang WH, Abeydeera LR, Cantley TC, Day BN, 1997: Effects of oocyte maturation media on development of pig embryos produced by in vitro fertilization. J Reprod Fertil **111,** 101–108.

Wang WH, Day BN, Wu GM, 2003: How does polyspermy happen in mammalian oocytes? Microsc Res Technol **61,** 335–341.

Xu X, Seth PC, Harbison DS, Cheung AP, Foxcroft GR, 1996: Semen dilution for assessment of boar ejaculate quality in pig IVM and IVF systems. Theriogenology **46,** 1325–1337.

Submitted: 26.07.2007

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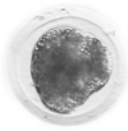

Artículo 5

In vitro maturation of porcine oocytes with retinoids improves embryonic development

Running Title: Retinoids improve embryonic development

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ABSTRACT

The effect of retinoid metabolite administration during in vitro maturation (IVM) on oocyte maturation, in vitro fertilization (IVF) parameters and embryo development was examined. Varying concentrations of 9-*cis* retinoic acid (RA; 0, 5, 50 and 500nM; Experiment 1) and all-*trans* retinol (ROH; 0, 125, 1250 and 12500nM; Experiment 2) were included. Cumulus-oocyte complexes were matured in vitro and

inseminated with frozen-thawed spermatozoa. Presumptive zygotes were cultured for 16h to assess IVF parameters or for 7 days to assess embryo development and quality. In experiment 1, oocyte maturation rates were significantly decreased ($p<0.001$), with values below 5%, when the highest concentration of RA (500nM) was used, whereas 5 and 50nM RA had no effect compared with the control. The 5nM RA treatment improved blastocyst development rate ($p<0.001$). In experiment 2, oocyte maturation rate did not differ between 125nM, 1250nM ROH and the control, but 12500nM ROH was deleterious. Penetration rate was lower for 1250 than for 125 nM ROH or the control, but blastocyst formation rate did not differ among the three maturation treatments. In conclusion, 5nM RA in the IVM medium significantly increased blastocyst formation rate, suggesting that RA may play an important role during IVM.

Keywords: all-*trans* retinol, 9-*cis* retinoic acid, In vitro Fertilization, Porcine oocytes.

INTRODUCTION

With growing interest in the generation of embryonic stem cells for the production of transgenic animals and the study of developmental gene regulation, there is an increasing reliance on the in vitro fertilization (IVF) laboratory to maximize embryo viability and quality. However, the in vitro production of porcine embryos has been limited by low rates of development to the blastocyst stage (Abeydeera and Day 1997; Kikuchi et al. 2002) and their poor quality in comparison with blastocyst produced in vivo (Wang et al. 1999). The low quality of oocytes after in vitro maturation (IVM) (Funahashi et al. 1997; Nagai 2000; Kikuchi et al. 2002), increased

polyspermy after IVF (Niwa 1993; Abeydeera and Day 1997; Wang et al. 1997a; Abeydeera 2002; Gil et al. 2004, 2007; Almiñana et al. 2005, 2007a,b) and poor developmental ability of embryos produced by IVM-IVF (Abeydeera 2001) are the main reasons for this limited performance, together with the unsuitability of in vitro culture systems (Kikuchi et al. 2002).

Successful nuclear maturation of oocytes in vitro is obtained with a high level of repeatability in most current porcine IVF systems. However, while a large proportion of oocytes reach metaphase II after IVM, the IVM environment may not support adequate cytoplasmic and molecular maturation (Sirard et al. 2006), which is required to prepare the oocyte for post-fertilization events, allowing the oocyte to reach the blastocyst stage. Compared to in vivo maturation, IVM conditions are simple and materially limited, which can profoundly affect the maturation status of oocyte. For this reason, improved culture conditions will be essential to obtain consistently successful and reliable oocyte maturation (both cytoplasmic and nuclear), leading to dramatically improved efficiency of in vitro embryo production.

There is growing evidence of the essential role of retinol and its metabolites, all-*trans* retinol (ROH) and 9-*cis* retinoic acid (RA), in cell growth, differentiation and embryonic development under in vivo and in vitro conditions (Shaw et al. 1995; Hidalgo et al. 2003). Both ROH and RA enter the cell nucleus and are able to activate retinoic acid receptors (RAR), whereas retinoid X receptors (RXR) are activated only by RA (Mangelsdorf et al. 1992; Chambon 1996). RAR-RXR heterodimers are the functional units transducting the retinoid signal at the gene level. The beneficial effects of ROH and RA have been demonstrated in bovine oocytes, especially on cytoplasmic

75 competence after in vitro maturation (Duque et al. 2002; Gomez et al. 2003; Ikeda et al.
76 unpublished data) and embryonic development and quality (Duque et al. 2002; Hidalgo
77 et al. 2003; Gomez et al. 2003; Lima et al. 2006; Livingston et al. 2004). In spite of the
78 beneficial effects of retinol, a suitable dosage for the different retinol metabolites has
79 yet to be determined, since both excessive and inadequate exposure to retinol and its
80 metabolites can be detrimental for mouse and bovine embryo development (Sporn and
81 Roberts 1991; Huang et al. 2006). However, to the best of our knowledge, there have
82 been no studies on the effects of different doses of retinol during IVM on the yield of
83 porcine embryos.
84
85 The objective of the present study was to evaluate the effect of both retinol
86 metabolites supplementation at different concentrations in the IVM medium on IVM
87 and IVF parameters and on in vitro embryo development quality.
88
89 MATERIALS AND METHODS
90
91 Culture media
92
93 The medium used for the collection and washing of cumulus-oocyte complexes
94 (COCs) was Dulbecco´s Phosphate-Buffered Saline (DPBS) medium composed of
95 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO4 and 1.46 mM $CaCl_2$-$2H_2O$
96 supplemented with 4 mg/mL bovine serum albumin (BSA; [fraction V]), 0.34 mM
97 sodium pyruvate, 5.4 mM D-glucose and 70 µg/ml Kanamycin (mDPBS). The oocyte
98 maturation medium was BSA-free North Carolina State University (NCSU)-23 (Peters
99 and Wells 1993) supplemented with 10% (v:v) porcine follicular fluid (pFF), 0.1

mg/mL cysteine and 10 ng/mL epidermal growth factor (EGF). The basic medium used for fertilization was essentially the same as that used by Abeeydera and Day (1997). This medium, designated as modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris (crystallized free base) 11 mM glucose, 5 mM sodium pyruvate, and was supplemented with 2mM caffeine and 0.2% BSA (fraction V; A 7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was a sequential medium based in NCSU-23 supplemented with 0.4% BSA.

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain).

Recovery and IVM of Cumulus-Oocyte Complexes

Ovaries were obtained from prepuberal gilts at a local slaughterhouse and transported to the laboratory at 35ºC within 1 h after collection in 0.9% NaCl containing 70 µg/mL Kanamycin. After the ovaries were washed three times in NaCl solution, follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle connected to a 10 ml disposable syringe. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times in maturation medium and matured in a 4-well multidish (Nunc, Roskilde, Denmark) containing 150-200 COCs per well in 500 µl of maturation medium supplemented with 10 IU/ml eCG (Folligon, Intervet International B.V., Boxmeer, The Netherlands) and 10 IU/ml hCG (Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain) for 20-22 h and then for another 20-22 h in

maturation medium without hormones. Oocyte maturation was carried out under mineral oil at 39ºC in an atmosphere of 5% CO_2 in air.

In vitro fertilization

After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium by vortexing for 2 min, 1660 rounds/min. Oocytes were washed twice in maturation medium and three times in pre-equilibrated fertilization medium. Groups of 50 denuded oocytes were then placed in 50 µl drops of fertilization medium in a 35 x 10 mm Petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, USA) under mineral oil and held at 39ºC in an atmosphere of 5% CO_2 in air for about 30 min until addition of spermatozoa.

For IVF, 100 µl of thawed semen were washed three times by centrifugation at 1900 x g for 3 min in mDPBS. The resulting pellet was resuspended in fertilization medium and after appropriate dilution, 50 µl of this sperm suspension was added to a 50 µl drop of fertilization medium containing the oocytes. The spermatozoa:oocyte ratio was 1000:1. The gametes were coincubated at 39ºC in a humidified atmosphere of 5% CO_2 in air for 6 h.

In vitro culture

Presumptive zygotes were removed from the fertilization medium and washed three times in pre-equilibrated embryo culture medium. Subsequently, they were transferred to a 4-well multidish (50 zygotes per well), each well containing 500 µl of

149 the same medium under mineral oil, and cultured at 39ºin 5% CO_2 in air for 16 h to
150 assess fertilization parameters or for 7 days to assess embryo development. Presumptive
151 zygotes were cultured for the first 2 days (Day 0 = day of fertilization) in glucose free
152 NCSU-23 supplemented with 0.33mM pyruvate and 4.5mM lactate. Following 48 h of
153 culture, all presumptive zygotes were removed and cultured subsequently in fresh
154 NCSU-23 medium containing 5.5 mM glucose until Day 7.
155
156 Assessment of maturation, sperm penetration and embryo development
157
158 To evaluate maturation and fertilization parameters, oocytes and presumptive
159 zygotes were mounted on slides, fixed in a solution of acetic acid:ethanol (1:3) for 48-
160 72 h at room temperature, stained with 1% lacmoid in 45% (v/v) acetic acid and
161 examined under a phase-contrast microscope at a magnification of 400 x. Maturation
162 rate was assessed at 44h of IVM. Oocytes with chromosomes at metaphase-II and an
163 extruded polar body were considered mature. Fertilization parameters were evaluated
164 16-18 h after insemination. Oocytes were considered penetrated when they contained
165 one or more swollen sperm heads and/or male pronuclei, with their corresponding
166 sperm tails, and two polar bodies. Degenerated oocytes and oocytes with a broken
167 oolemma or abnormal cytoplasmic appearance were not counted. The fertilization
168 parameters evaluated were: penetration (number of oocytes penetrated /total oocytes
169 matured), monospermy (number of oocytes containing only one male pronucleus/total
170 penetrated), number of spermatozoa/oocyte (number of spermatozoa in penetrated
171 oocytes), and efficiency of fertilization (number of monospermic oocytes/total
172 inseminated).
173

To examine the ability of embryos to develop to the blastocyst stage in vitro, presumptive zygotes were cultured for 7days. On day 2, cleavage rate (number of embryos cleaved/total cultured) and on day 7, blastocyst formation (number of blastocyst/ total cultured) were evaluated under a stereomicroscope. An embryo which had cleaved to the two-cell stage or beyond was counted as cleaved and an embryo with a clear blastocele was defined as a blastocyst. The total cell number, as an indicator of embryo quality, was evaluated by mounting each blastocyst on a slide in 4µL of a dilution of Glycerol-DPBS (3:1) containing 10 µg/mL Hoechst-33342, followed by examination using fluorescence microscopy.

Experimental design

Experiment 1. Effect of 9-*cis* retinoic acid (RA) during in vitro maturation on IVM, IVF parameters and in vitro embryo development.

In an attempt to improve in vitro oocyte maturation and in vitro embryo development, various concentration of RA (0, 5, 50 and 500nM) were added to the maturation medium. A total of 2161 in vitro matured oocytes, from five replicates, were used in this experiment.

Experiment 2. Effect of all-*trans* retinol (ROH) during in vitro maturation on IVM, IVF parameters and in vitro embryo development

This experiment evaluated the effect of ROH on porcine in vitro maturation and in vitro embryo production. Various concentrations of ROH (0, 125, 1250 and

12500nM) were tested. A total of 2141 in vitro matured oocytes from five replicates were used in this experiment.

Statistical analysis

All data editing and statistical analyses were performed by SPSS, version 11.5 (SPSS Inc., Chicago, III). Data from all replicates were assessed by analysis of variance using the MIXED procedure according to a statistical model including the fixed effect of RA (Experiment 1) and ROH (Experiment 2) concentration and the random effect of replicate in each experiment. Maturation, penetration and monospermic rates, efficiency data and blastocyst formation were modelled according to the binomial model of parameters as described by Fisz (1980) before analysis. When analysis of variance showed a significant effect, values were compared using the Bonferroni test. The threshold for significance was set at $p< 0.05$. Results are expressed as least squares means ± SEM.

RESULTS

Experiment 1. Effect of RA during in vitro maturation on IVM, IVF parameters and in vitro embryo development.

Oocyte maturation rates were significantly decreased ($p<0.001$), with values below 5%, when the highest concentration of RA (500nM) was used (Figure 1). No effect of 5nM and 50nM of RA was observed on *in vitro* oocyte maturation compared to the control. However, maturation rates were higher for 5 nM than 50 nM RA ($p<0.05$).

224

225 Penetration rate, monospermy rate and mean number of spermatozoa per oocyte
226 were not influenced by RA treatment (Table 1). There were no differences in the
227 efficiency of IVF between the control and 5 nM RA but efficiency decreased for 50
228 ($p<0.05$) and 500 nM RA ($p<0.001$) compared to the control. The low efficiency of IVF
229 for the highest concentration of RA (0.7±3.8) was due to the low maturation rate
230 (2.0±2.7).

231

232 Data on embryo development are presented in Figure 2. There were no
233 differences in cleavage rate between the control, 5 or 50nM RA but the 5nM RA
234 treatment significantly increased the rate of blastocyst formation ($p<0.001$). The total
235 cell number per blastocyst was not different among treatments and ranged from
236 25.14±1.52 to 27.82±1.44. No embryo cleavage and blastocyst formation were observed
237 when oocytes were exposed to the highest concentration of RA (500 nM) during IVM.

238

239 Experiment 2. Effect of ROH during in vitro maturation on IVM, IVF
240 parameters and in vitro embryo development

241

242 In this experiment, maturation rates did not differ between the control, 125 nM
243 and 1250 nM ROH treatments, but 12500 nM ROH was deleterious (Figure 3). No
244 oocytes matured with 12500 nM ROH, so it was not possible to evaluate IVF
245 parameters and embryo development.

246

247 Penetration rate was significantly lower for 1250 than 125 nM ROH or the
248 control, but monospermy rate, the mean number of spermatozoa per oocyte and the
249 efficiency of IVF were not influenced by the ROH treatments (Table 2).
250
251 The addition of 125nM of ROH resulted in a significant increase ($p<0.001$) in
252 the percentage of cleaved embryos (Figure 4). However, blastocyst formation rate and
253 total cell number were unaffected by ROH treatment, apart from a depression in the
254 percentage of blastocysts after maturation of oocytes with 1250nM ROH (Figure 4).
255
256 DISCUSSION
257
258 While retinol metabolites have been recommended as important components of
259 IVM media to improve cytoplasmic maturation and embryo development, both
260 beneficial and detrimental effects have been described for bovine embryo development
261 in vitro (Duque et al. 2002; Gomez et al. 2003; Hidalgo et al. 2003; Lima et al. 2006).
262 To the best of our knowledge, there are no reports on the effects of retinoids on the
263 developmental ability of porcine oocytes.
264
265 Too much or too little exposition to retinoids, at the wrong stage or at the wrong
266 time, can adversely affect embryo development (Sporn and Roberts 1991; Huang et al.
267 2006; Rodríguez et al, 2007). In the present study, the highest concentrations of both
268 metabolites (500 nM for RA and 12500 nM for ROH) were cytotoxic because they
269 prevented oocytes from achieving nuclear maturation. This detrimental effect could be
270 related to a retinoid imbalance that has been shown to be highly correlated with
271 developmental abnormalities (Morris-Kay and Ward 1999). In support of this

hypothesis, high concentrations of retinoids during IVM, such as 500nM of RA (Gomez et al. 2003) and 100 µM ROH (Livingston et al. 2004) have been shown to be detrimental for bovine oocytes. Contrary to these findings, Lima et al. (2006) reported that addition of 500nM of RA to bovine IVM medium enhanced blastocyst formation. A possible explanation for these discrepancies could be the different IVM conditions used by these authors, especially the different hormone supplementation regimes. Thus, Gomez et al. (2003) suggested that the detrimental effect of overexposure to retinoids, or its neutral effect observed during IVM, might depend on an interaction between retinoids and FSH. Moreover, it has been demonstrated that retinol decreases FSH-induced expression of LH receptor in porcine granulosa cells (Hattori et al. 2000).

Taking into consideration the importance of an accurate dosage, a range of retinoid concentrations was examined during IVM in the present study. The RA and ROH concentrations were chosen based on those used in previous reports in other species, in order to establish a suitable dosage for porcine IVM. Our results showed no effect of 5 nM and 50 nM of RA on in vitro nuclear oocyte maturation. However, the presence of 5 nM of RA showed a tendency ($p=0.18$) to improve maturation rates compared to control and was significantly better than the 50 nM treatment. When ROH was tested during IVM, maturation rates did not differ between the control and 125 nM or 1250 nM ROH.

These results are in contrast to those obtained with bovine oocytes, where retinoids were reported to improve nuclear maturation (Bortolotto, unpublished data). There could be species-specific differences in susceptibility to retinoids, with varied requirements during IVM in order to protect oocytes from detrimental effect on the one

297 hand and from the epigenetic influences of retinol on the other, as has been reported by
298 several authors (e.g. Gomez et al. 2003; Huang et al. 2006). The different concentrations
299 of retinoids used between studies also could have been responsible for such
300 discrepancies, knowing that the effects of these compounds are dependent on their
301 concentration (Gomez et al. 2003; Livingston et al. 2004; Lima et al. 2006).
302
303 In the present study, a significant increase in blastocyst development was
304 obtained after treatment of oocytes with 5 nM RA. However, a neutral or detrimental
305 effect was observed when 50 nM RA or 125 and 1250 nM ROH were used. The better
306 developmental competence achieved by oocytes, and consequently increases in
307 blastocyst formation, after exposure to 5 nM RA during IVM, could be related to more
308 complete granular migration in the matured oocyte cytoplasm induced by RA, as
309 suggested to occur in bovine oocytes (Gomez et al. 2003). This migration provides a
310 block to polyspermy once migrated cortical granules (CG) have been released (Wang et
311 al. 1997b; Nagano et al. 1999). It is well known that CG play an important role in the
312 block to polyspermy in mammalian oocytes (Yanagimachi 1994). Polyspermy is one of
313 the main unresolved problems of porcine IVF that greatly limits the production of useful
314 pig embryos. Based on the hypothesis that the benefits of RA would be reflected in
315 improved granular migration, an increase in monospermy rates should have been
316 obtained in our study. However, the proportion of monospermic oocytes was not
317 influenced by RA treatment. Moreover, exposure of oocytes to RA did not affect
318 penetration rate and the mean number of spermatozoa per oocyte. This indicates that the
319 improved developmental competence of oocytes after RA treatment was not associated
320 with a reduction in polyspermy, at least under our experimental conditions.
321

A possible explanation for the increased blastocyst development after 5 nM RA treatment is protection from oxidative damage, which is a major cause of in vitro embryonic wastage (Guerin et al. 2001). Previous studies have shown that retinoids participate in a biological antioxidant network, and have been implicated as important regulators of redox signalling pathways (Olson 1993; Imam et al. 2001, Ikeda et al. 2005). Furthermore, it has been reported that retinoids protect against oxidative damage by maintaining adequate endogenous competency and levels of antioxidants which are essential for oocyte maturation, fertilization and embryonic development (Guerin et al. 2001). However, this beneficial effect was not reflected in embryo cleavage or embryo quality in the present study, in contrast with bovine embryos, where increased cell numbers and proportions of cells allocated to the inner cell mass have been reported (Duque et al. 2002). These differences might be dependent on the amount of triglyceride stored in the pig oocyte, higher than that in bovine (Genicot et al, 2005; McEvoy et al, 2000). Sterification to fatty acids, particularly palmitate, is the preferential form for retinol storage in cells, and palmitate is the most abundant fatty acid in oocytes of domestic species, including the pig (McEvoy et al, 2000; Kim et al, 2001). Therefore, it is feasible that the pig oocyte contains larger amounts of retinol than the bovine oocyte. As retinol could be made active through conversion to retinoic acid, such as detected in bovine oocytes and embryos (Rodriguez et al, 2006; Gómez et al, 2006), this could lead to differences in endogenous retinoid activity. Interference of exogenous compounds with endogenous retinoid may explain differences between species.

When ROH treatments were evaluated, penetration rate was significantly lower for 1250 than 125 nM ROH or the control. Monospermy rate and the mean number of spermatozoa per oocyte were not affected by the ROH treatments. Although treatment

of oocytes with 125nM ROH resulted in better embryo cleavage compared to the control, this enhancement was not reflected in blastocyst development. Moreover, the number of cells per blastocyst was not influenced by any of the ROH treatments. These results are in disagreement with those obtained in bovine IVF, where it was reported that 5000 nM ROH during IVM increased embryonic development to the blastocyst stage (Livingston et al. 2004). It may be that different concentrations of this metabolite to those used in the present study are required during porcine IVM to achieve improved embryo development.

In conclusion, the present study showed that RA at a concentration of 5nM in the IVM medium significantly increased blastocyst formation. These data suggest that inclusion of RA in IVM media used for current IVF protocols could improve the yield of porcine IVP embryos. Additional studies are needed to elucidate the possible mechanisms by which RA influences the developmental capacity of porcine oocytes.

ACKNOWLEDGMENTS

The authors wish to thank Professor W.M.C. Maxwell for helpful discussion and critical reading of the manuscript. The SENECA foundation of Murcia is acknowledged for funding a grant for C. Almiñana (00197/BPS/04). This study was supported by CICYT-FEDER (AGL2004-07546) and CDTI (04-0231) projects.

REFERENCES

Abeydeera, L. R., and Day, B. N. (1997). Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol Reprod*. **57,** 729-34.

Abeydeera, L. R.(2001). In vitro fertilization and embryo development in pigs. *Reprod*. **58**, 159-73.

Abeydeera, L. R. (2002). In vitro production of embryos in swine. *Theriogenology* **57**, 256-73.

Almiñana, C., Gil, M. A., Cuello, C., Roca, J., Vazquez, J. M., and Martinez, E. A. (2005) Adjustments in IVF system for individuals: value of additives and time of sperm-oocyte co-incubation. *Theriogenology* **64,** 1783-1796.

Almiñana, C., Gil, M. A., Cuello, C., Parrilla, I., Roca, J., Vazquez, J.M., and Martinez, E. A. (2007a). Effects of ultrashort gamete co-incubation on porcine in vitro fertilization. *Anim. Reprod. Sci.* doi:10.1016/j.anireprosci.2007.05.017

Almiñana, C., Gil, M.A., Cuello, C., Caballero, I., Roca, J., Vazquez, J. M., and Martinez, E. A. (2007b). In vitro fertilization (IVF) in straws and a short gamete coincubation time improves the efficiency of porcine IVF. *Reprod. Domestic Anim. (Accepted)*

Chambon, P . (1996). A decade of molecular biology of retinoic acid receptors FASEB Journal. **10,** 940-954.

Duque, P., Diez, C., Royo, L., Lorenzo, P.L., Carneiro, G., Hidalgo, C.O., Faca,l N., and Gomez, E. (2002) Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. *Hum. Reprod.* **17,** 2706-14.

Fisz, M. (1980). Some probability distribution. In "Probability theory and mathematical statistics". (Eds De Krieger R) pp. 129-74. (Malabar, FL, USA: John widely and sons)

Funahashi, H., Cantley, T. C., and Day, B. N. (1997). Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol. Reprod.* **57**, 49-53.

Genicot G, Leroy JL, Soom AV, Donnay I. (2005) The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology. 63:1181-1194.

Gil, M. A., Ruiz, M., Vazquez, J. M., Roca, J., Day, B. N., and Martinez, E. A. (2004). Effect of short period of sperm-oocyte coincubation during in vitro fertilization on embryo development in pigs. *Theriogenology* **62**, 544-52.

Gil, M.,A., Almiñana, C., Cuello, C., Parrilla, I. , Roca, J., Vazquez, J. M., and Martinez E. A. (2007). Brief coincubation of gametes in porcine in vitro

fertilization: role of sperm:oocyte ratio and post-coincubation medium. *Theriogenology* **67**, 620-626.

Gomez, E., Royo, L. J., Duque, P., Carneiro, G., Hidalgo, C., Goyache, F., Lorenzo, P. L., Alvarez, I., Facal, N., and Diez, C. (2003). 9-cis-retinoic acid during in vitro maturation improves development of the bovine oocyte and increases midkine but not IGF-I expression in cumulus-granulosa cells. *Mol. Reprod. Dev.* **66**, 247-55.

Gomez E, Caamano J, Rodriguez A, De Frutos C, Facal N, Diez C. Bovine early embryonic development and vitamin A. Reprod Domest Anim. 2006 41:63-71.

Guerin, P., El Mouatassim, S., and Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod. Update* **7**, 175-189.

Hattori, M., Takesue, K., Nishida, N., Kato, Y., and Fujihara, N. (2000). Inhibitory effect of retinoic acid on the development of immature porcine granulosa cells to mature cells. *J. Mol. Endocrinol.* **25**, 53-61.

Hidalgo, C. O., Diez, C., Duque, P., Facal, N., and Gomez, E. (2003). Pregnancies and improved early embryonic development with bovine oocytes matured in vitro with 9-cis-retinoic acid. *Reproduction.* **125**, 409-16.

444 Huang, F.J., Hsuuw, Y. D., Lan, K. C., Kang, H. Y., Chang, S. Y., Hsu, Y. C., and
445 Huang, K. E. (2006). Adverse effects of retinoic acid on embryo development
446 and the selective expression of retinoic acid receptors in mouse blastocysts.
447 *Hum. Reprod*. **21**, 202-9.
448
449 Ikeda, S., Kitagawa, M., Imai, H., and Yamada, M. (2005). The roles of vitamin A for
450 cytoplasmic maturation of bovine oocytes. *J. Reprod. Dev.* **51,** 23-35.
451
452 Imam, A., Hoyos, B., Swenson, C., Levi, E., Chua, R., Viriya, E., and HammerlIng, U.
453 (2001). Retinoids as ligands and coactivators of protein kinase C alpha. *FASEB J*
454 **15**, 28-30.
455
456 Kikuchi, K., Onishi, A., Kashiwazaki, N., Iwamoto, M., Noguchi, J., Kaneko, H., Akita,
457 T., and Nagai, T. (2002). Successful piglet production after transfer of
458 blastocysts produced by a modified in vitro system. *Biol. Reprod.* **66,** 1033-41.
459
460 Kim JY, Kinoshita M, Ohnishi M, Fukui Y (2001) Lipid and fatty acid analysis of fresh
461 and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction.
462 122:131-138.
463
464 Lima, P. F., Oliveira, M. A., Santos, M. H., Reichenbach, H. D., Weppert, M., Paula-
465 Lopes, F. F., Neto, C. C., and Goncalves, P.B. (2006). Effect of retinoids and
466 growth factor on in vitro bovine embryos produced under chemically defined
467 conditions. *Anim. Reprod. Sci.* **95,** 184-92.
468

469 Livingston, T., Eberhardt, D., Edwards, J. L., and Godkin, J. (2004). Retinol improves
470 bovine embryonic development in vitro. *Reprod. Biol. Endocrinol*. **21**, 2:83.
471
472 Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E.,
473 Kakizuka, A. and Evans, R. M. (1992). Characterization of three RXR genes that
474 mediate the action of 9-*cis* retinoic acid. *Genes and Development* **6**, 329-344.
475
476 McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JS, Speake BK (2000) Fatty acid
477 composition of lipids in immature cattle, pig and sheep oocytes with intact zona
478 pellucida. J. Reprod. Fertil. 118:163-70.
479
480 Morris-Kay, G. M., and Ward, S. J. (1999). Retinoids and mammalian development. *Int.*
481 *Rev. Cytol*. **188**, 73-131.
482
483 Nagano, M., Takahashi, Y., and Katagiri, S. (1999). In vitro fertilization and cortical
484 granule distribution of bovine oocytes having heterogeneous ooplasm with dark
485 clusters. *J. Vet. Med. Sci.* **61**, 531-5.
486
487 Nagai, T. (2000). The improvement of in vitro maturation systems for bovine and
488 porcine oocytes. *Theriogenology* **55**, 1291-1301.
489
490 Niwa, K. (1993). Effectiveness of in vitro maturation and in vitro fertilization
491 techniques in pigs. *J. Reprod. Fertil. Suppl*. **48**, 49-59.
492

493 Olson, J. A. (1993). Vitamin A and carotenoides as antioxidants in a phishiological
494 context. *J. Nutr. Sci. Vitaminol.* (Tokyo) **39**, S57-S65.
495
496 Petters, R. M., and Wells, K. D. (1993). Culture of pig embryos. *J Reprod Fertil Suppl*.
497 **48**, 61-73.
498
499 Rodríguez A, Díez C, Ikeda S, Royo LJ, Caamaño JN, Alonso-Montes C, Goyache F,
500 Alvarez I, Facal N and Gómez E. Retinoids during the in vitro transition from
501 bovine morula to blastocysts. Human Reproduction 2006;21:2149-2157.
502
503 Rodríguez A, Díez C, Caamaño JN, de Frutos C, Royo LJ, Muñoz M, Ikeda S, Facal N,
504 Álvarez-Viejo M and Gómez E. (2007) Retinoid receptor-specific agonists
505 regulate bovine in vitro early embryonic development, differentiation and
506 expression of genes related to cell cycle arrest and apoptosis. Theriogenology,
507 doi:10.1016/j.theriogenology.2007.08.007
508
509 Shaw, D. W., Farin, P. W., Washburn, S.P., and Britt, J. H. (1995). Effects of retinol
510 palmitate on ovulation rate and embryo quality in superovulated cattle.
511 *Theriogenology*. **44**, 51-58.
512
513 Sirard, M. A., Richard, F., Blondin, P., and Robert, C. (2006). Contribution of the
514 oocyte to embryo quality. *Theriogenology* **7**, 126-36.
515

516 Sporn, M. B., and Roberts, A. (1991). Interactions of retinoids and transforming growth
517 factor-beta in regulation of cell differentiation and proliferation. *Mol Endocrinol.*
518 **5**, 3-7.
519
520 Wang, W. H., Abeydeera, L. R., Cantley, T. C., and Day, B. N. (1997a). Effects of
521 oocyte maturation media on development of pig embryos produced by in vitro
522 fertilization. *J. Reprod. Fertil.* **111**, 101-8.
523
524 Wang, W., Hosoe, M., Li, R., and Shioya, Y. (1997b). Development of the competence
525 of bovine oocytes to release cortical granules and block polyspermy after
526 meiotic maturation. *Dev. Growth. Differ.* ***39***, 607-15.
527
528 Wang, W. H., Abeydeera, L. R., Han, Y. M., Prather, R. S., and Day, B. N. (1999).
529 Morphologic evaluation and actin filament distribution in porcine embryos
530 produced in vitro and in vivo. *Biol. Reprod.* **60**, 1020-8.
531
532 Yanagimachi, R. (1994). Mammalian fertilization. In "The Physiology of
533 Reproduction". (Eds Knobil, E. Neil, J.D.) pp. 261-268. (New York: Raven
534 Press, LTD)
535
536

Table1. Effect of 9-*cis* retinoic acid (RA) during in vitro maturation on IVF parameters of porcine oocytes.

Treatment Concentration of RA (nM)	No. Oocytes	Penetration (%)	Monospermy (%)	EO	Efficiency (%)
0	179	91.4±3.5	54.0±5.7	1.84±0.19	45.4±3.4 [a]
5	206	84.6±3.4	53.2±5.5	1.80±0.18	41.8±3.2 [ab]
50	164	87.2±3.7	39.9±5.9	1.93±0.19	30.7±3.5 [b]
500	144	96.6±23.4	55.2±35.3	2.89±0.80	0.7±3.8 [c]

[a,b,c] Different superscripts within the same column represent a significant difference (at least $p<0.05$).

Penetration: number of oocytes penetrated/total oocytes matured.

Monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated.

EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes.

Efficiency: number of monospermic oocytes/total inseminated.

Table 2. Effect of All-*trans*-retinoic (ROH) during in vitro maturation on IVF parameters of porcine oocytes.

Treatment Concentration of ROH (nM)	No. Oocytes	Penetration (%)	Monospermy (%)	EO	Efficiency (%)
0	165	89.2±2.8[a]	50.2.0±4.8	1.72±0.12	42.5±3.4
125	169	85.7±2.9 [a]	47.2±4.9	1.78±0.12	38.8±3.5
1250	170	72.2±3.0[b]	52.8±5.4	1.87±0.13	34.3±3.4
12500	164				

[a,b] Different superscripts within the same column represent a significant difference (at least $p<0.05$).

Penetration: number of oocytes penetrated/total oocytes matured.

Monospermy: number of oocytes containing only one sperm head or one male pronuclei/total penetrated.

EO (number of spermatozoa/oocyte): number of spermatozoa in penetrated oocytes.

Efficiency: number of monospermic oocytes/total inseminated.

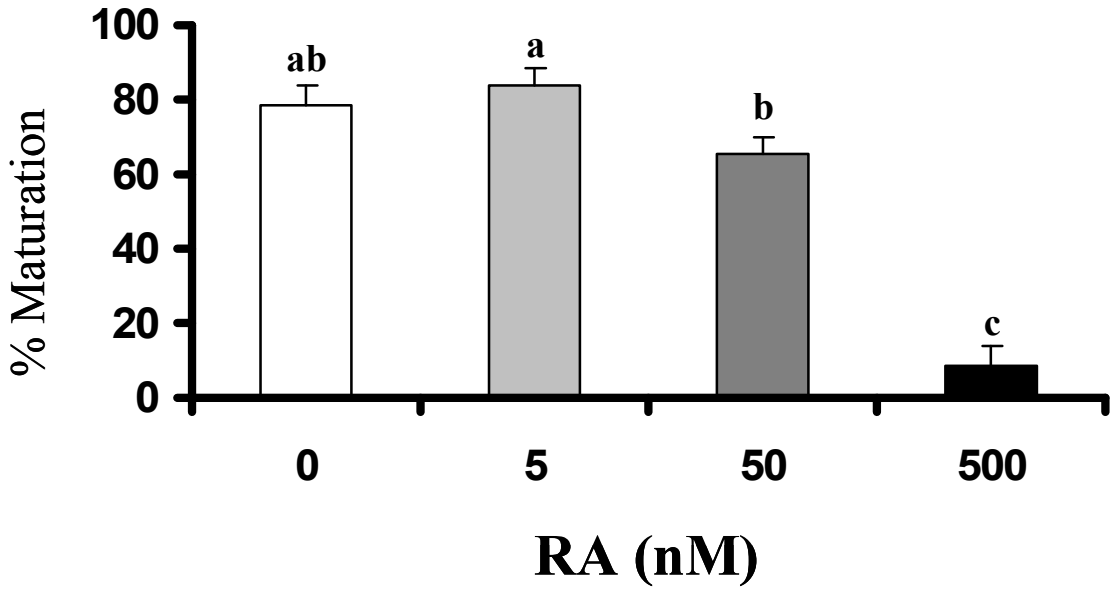

Figure 1. Effect of 9-*cis* retinoic acid (RA) on in vitro maturation rate of porcine oocytes. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times and matured in maturation medium (NCSU-23) supplemented with 0, 5, 50 or 500nM RA. Maturation rates were assessed after 44h of IVM. Oocytes with chromosomes at metaphase-II and an extruded polar body were considered mature. $^{a,b,c}(p<0.05)$

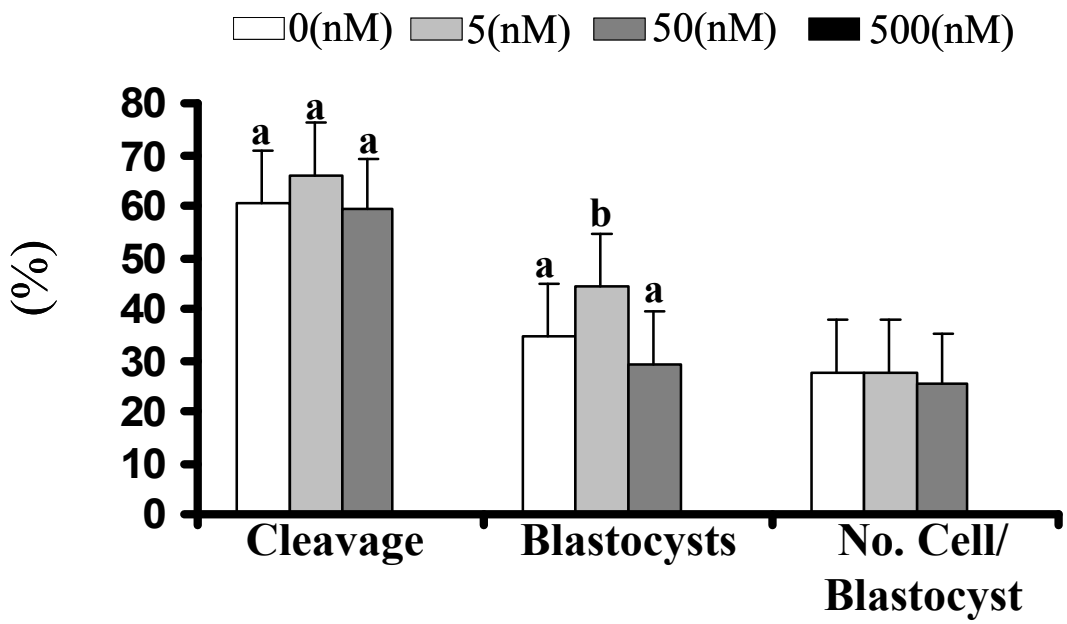

Figure 2. Effect of 9-*cis* retinoic acid during in vitro maturation on in vitro development of porcine embryos. Oocytes were matured in maturation medium (NCSU-23) supplemented with 0, 5, 50 or 500nM RA for 44 h and were fertilized with frozen-thawed spermatozoa (1000 sperm/oocyte). After 6 h coincubation, presumptive zygotes from all groups were cultured in NCSU-23 medium for 7 days to assess embryo development. $^{a,b}(p<0.05)$ (% Cleavage: percentage of embryos cleaved/total oocytes inseminated; % Blastocysts: percentage of blastocysts/total oocytes inseminated). The total cell number was evaluated by mounting each blastocyst on slides in 4µL of a dilution of Glycerol-DPBS (3:1) containing 10 µg/mL of Hoechst-33342 followed by examination using fluorescence microscopy.

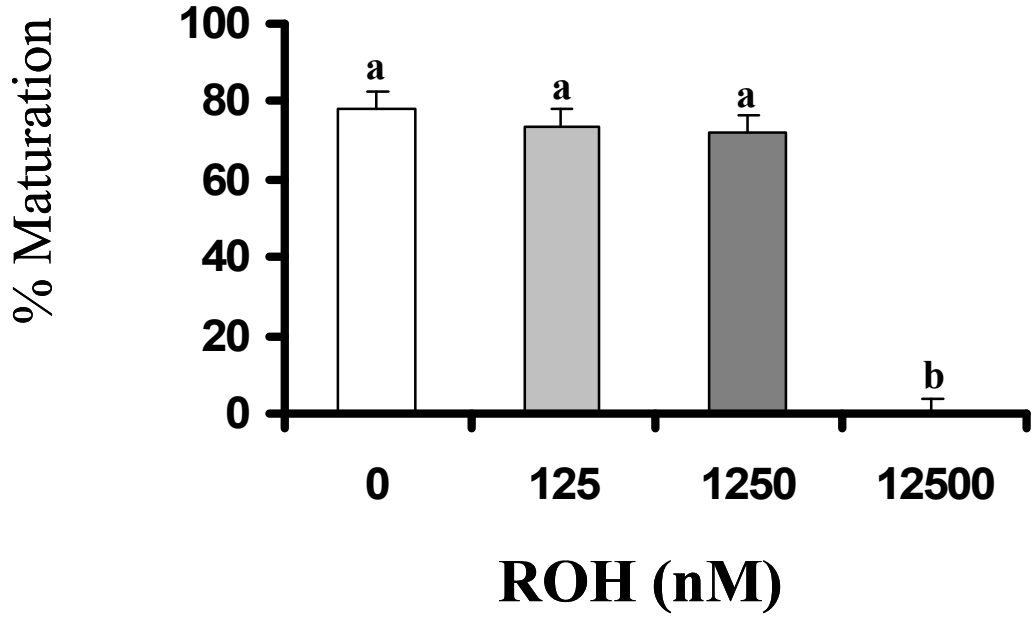

Figure 3. Effect of All-trans-retinoic (ROH) during in vitro maturation on maturation rates of porcine oocytes. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times and matured in maturation medium (NCSU-23) supplemented 0, 125, 1250 or 12500nM ROH.. Maturation rates were assessed before fertilization after 44h of IVM. Oocytes with chromosomes at metaphase-II and an extruded polar body were considered mature. [a,b]($p<0.05$)

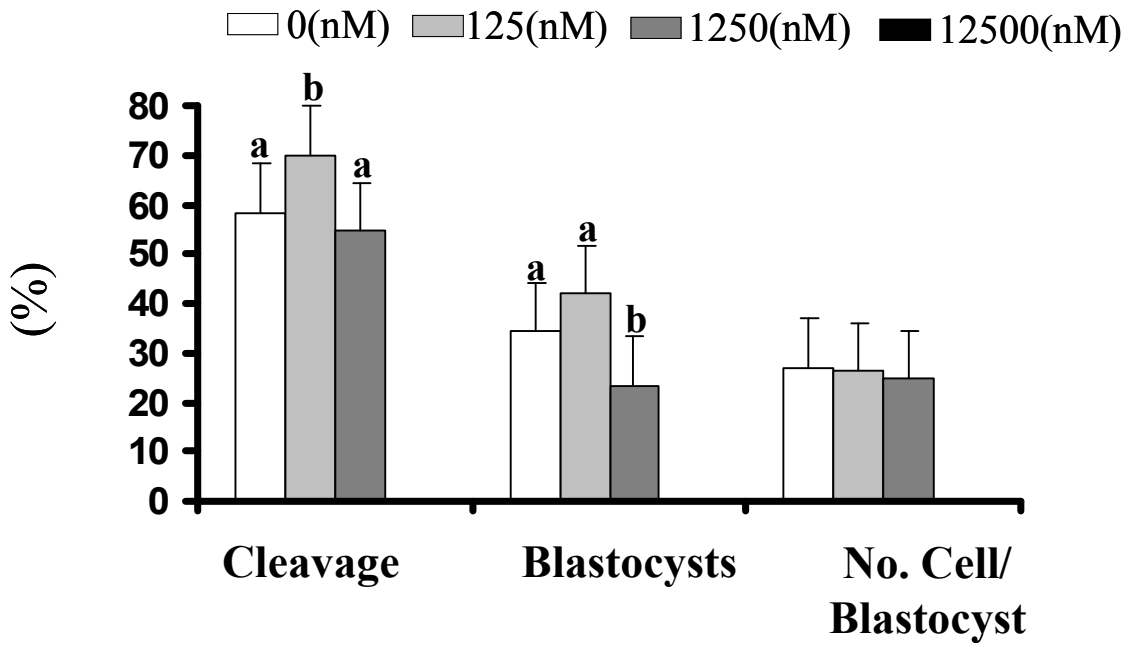

Figure 4. Effect of All-*trans*-retinoic (ROH) during in vitro maturation on in vitro development of porcine embryos. Oocytes were matured in maturation medium supplemented with 0, 125, 1250 or 12500nM ROH for 44 h and were fertilized with frozen-thawed spermatozoa (1000 sperm/oocyte). After 6 h coincubation, presumptive zygotes from all groups were cultured in NCSU-23 medium for 7 days to assess embryo development. $^{a,b}(p<0.05)$ (% Cleavage: percentage of embryos cleaved/total oocytes inseminated; % Blastocysts: percentage of blastocysts/total oocytes inseminated). The total cell number was evaluated by mounting each blastocyst on slides in 4µL of a dilution of Glycerol-DPBS (3:1) containing 10 µg/mL of Hoechst-33342 followed by examination using fluorescence microscopy.

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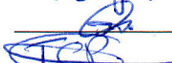
(3)Author Signature		Print Name	Cristina Cuello	Date	26/09/07
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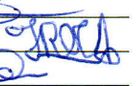
(5)Author Signature		Print Name	Jordi Roca	Date	26/09/07
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Conclusiones

Conclusiones 8

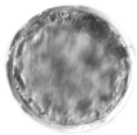
1. El tiempo de coincubación puede ser reducido a 10 minutos como estrategia para incrementar la eficiencia de la fecundación dependiendo del ratio espermatozoides:ovocito utilizado. La post-incubación de los ovocitos con los espermatozoides unidos a la zona pelúcida en un medio no apropiado para la FIV no mejora la eficiencia de la fecundación. Los espermatozoides unidos a la zona pelúcida requieren un máximo de 2 h en medio apropiado para penetrar los ovocitos (Artículo 1).

2. Las necesidades de los espermatozoides para la FIV en términos de aditivos del medio de fecundación y tiempo de coincubación varían entre verracos (Artículo 2).

3. Un tiempo de coincubación tan corto como 2 min es suficiente para obtener unas tasas de penetración, monospermia y una eficiencia de la FIV similares a las alcanzadas por los sistemas de FIV porcina actuales donde utilizan tiempos largos (6 h) de exposición (Artículo 3).

4. El sistema de FIV en pajuela junto con un corto tiempo de coincubación (10 min) aumenta la penetración monospérmica, mejora la eficiencia de la FIV y la calidad de los blastocistos en términos de número de células por blastocisto comparado con el sistema tradicional en microgota y 6 h de coincubación. Además este sistema permite el uso de un amplio rango de ratios espermatozoide:ovocito (Artículo 4).

5. La adición de 5 nM de *9- cis* ácido retinoico al medio de MIV aunque no mejora la tasa de maduración ni de fecundación en comparación con el grupo control, aumenta significativamente la formación de blastocistos (Artículo 5).



Resumen General

Resumen General 10

OBJETIVOS

En un intento de optimizar el sistema de fecundación *in vitro* (FIV) en la especie porcina estudiamos la influencia de distintas condiciones de maduración y de cocultivo de los gametos que podrían afectar la eficiencia de dicho sistema. Para ello se propusieron los siguientes objetivos:

1. Evaluar el efecto de diferentes ratios espermatozoide:ovocito durante la FIV utilizando un tiempo de coincubación corto (10 min) y diferentes periodos de post-coincubación en un medio no apropiado para la FIV sobre los parámetros de fecundación (Artículo 1).

2. Optimizar un protocolo de FIV para machos individuales, empleando como aditivos la cafeína, ácido hialurónico (AH) y la adenosina en el medio de fecundación y diferentes tiempos de coincubación de los gametos (Artículo 2).

3. Examinar el efecto de tiempos de coincubación ultracortos en la eficiencia de la fecundación *in vitro* porcina (Artículo 3).

4. Estudiar si la combinación de un sistema de FIV en pajuela con un tiempo de de coincubación corto podría ser una estrategia adecuada para disminuir la polispermia y mejorar la producción de blastocistos porcinos *in vitro* (Artículo 4).

5. Determinar el efecto de la administración de dos metabolitos del retinol, all-*trans* retinol y 9- *cis* retinoid acid, a diferentes concentraciones durante la maduración *in vitro* (MIV) sobre los parámetros de FIV y el desarrollo embrionario (Artículo 5).

METODOLOGÍA

MATERIAL

Material animal

Los ovocitos utilizados para la realización de la presente tesis se obtuvieron a partir de ovarios procedentes de cerdas prepuberales, con un peso comprendido entre 90 y 100 Kg, sacrificadas en un matadero industrial. El criterio de selección de los ovarios se basó en la presencia de folículos antrales de 3 a 6 mm de diámetro, eliminando aquellos que presentaban cuerpos lúteos o algún tipo de alteración morfológica macroscópica.

Se utilizó semen criopreservado procedentes de eyaculados de verracos híbridos, de fertilidad conocida, seleccionados previamente en base a la capacidad fecundante de sus espermatozoides y posterior desarrollo embrionario *in vitro* hasta el estadio de blastocisto.

Reactivos químicos y medios utilizados

Todos los medios, hormonas y suplementos utilizados en este trabajo se adquirieron de Sigma- Aldrich Co. (Alcobendas, Madrid, España), salvo que se indique lo contrario. Todos los medios se prepararon con agua bidestilada y purificada (purificador Elgastat UHQ ps, Elga) en un ambiente controlado (cámara de flujo laminar horizontal, BH-100 de Telstar). Los medios se esterilizaron con un equipo de filtración, Steritop (Millipore) (Sterivex GP, Millipore) mediante filtros de membrana de diámetro de poro de 0'22 µm. El pH (pH Crison 2000) y la osmolaridad (Microosmómetro Model 2-MO plus; Advanced Instruments Inc., USA) de cada medio fueron controlados en todos y cada uno de los ensayos realizados. Una vez preparados los medios se almacenaron a 5ºC y se utilizaron antes de los 15 días de su preparación. El medio utilizado para el transporte de los ovarios desde el matadero hasta el laboratorio fue solución salina (0'9% ClNa) suplementada con Kanamicina (70 µg/ml). El medio usado para la recolección, lavado y manipulación de los ovocitos fue PBSD (Dulbecco´s phosphate-buffered saline), al que se le añadió 4 mg/mL albúmina sérica bovina (BSA; [fracción V], A8022), 0'34 mM de piruvato sódico, 5'4 mM de glucosa y 70 µg/ml de Kanamicina (PBSDm) (Mattioli y cols., 1988a). El medio de maduración de los ovocitos fue el NCSU-23 (North Caroline State University), libre de BSA. En el momento de su utilización se enriqueció con 10% (v:v) de

fluido folicular porcino, 0'1 mg/mL de cisteína y 10 ng/mL de factor de crecimiento epidérmico, constituyendo el medio de maduración *in vitro*. Una vez suplementado, se depositó en las placas de cultivo. Posteriormente, se le añadió 10 IU/mL de eCG (Folligon, Intervet International B.V., Boxmeer, The Netherlands) y 10 IU/mL de hCG (Chorulon, Intervet International) durante las primeras 20-22 h de maduración. El medio básico usado para la fecundación fue el mismo que el usado por Abeydeera y Day (1997a) el TBMm (modified Tris-buffered médium). En el momento de su utilización, se enriqueció con 0'2% BSA ([fracción V], A7888, fracción inicial precipitada mediante alcohol frío) y con distintos suplementos de acuerdo al diseño experimental. Una vez suplementado, se depositó en las placas de cultivo. El medio de cultivo de embriones fue el NCSU-23, al que se le adicionó 0'4% BSA ([fracción V], A8022)) en el momento de su utilización.

MÉTODOS

Recolección y maduración de los complejos cúmulus-ovocito

Los complejos cúmulus-ovocito (COCs) fueron obtenidos de ovarios de hembras prepuberales en un matadero local y transportados al laboratorio en solución salina con Kanamicina a 34-37ºC, en la hora posterior a su recolección Después de lavar los ovarios tres veces en solución salina se procedió a la aspiración de los folículos, con ayuda de una aguja de 18 g unida a una jeringuilla de 10 ml. El líquido folicular con los ovocitos se introdujo en tubos de 15 ml para proceder a su sedimentación durante 10 minutos. Pasado este tiempo se eliminó el sobrenadante y el sedimento resultante, compuesto por ovocitos y células foliculares, se resuspendió en medio de lavado de los ovocitos, trasladando el contenido del tubo a placas de Petri, en las cuales se procedió a la identificación y selección de los ovocitos inmaduros con la ayuda de un estéreomicroscopio (SMZ-2T Nikon) a 60 aumentos. Sólo se utilizaron aquellos ovocitos rodeados por varias capas de células del cúmulus compactas y con un citoplasma uniformemente negro y granulado. Los COCs fueron lavados tres veces en medio de maduración y depositados en placas de 4 pocillos (Nunc, Roskilde, Denmark) durante el periodo de maduración. La gota de medio de maduración en cada pocillo fue de un volumen de 500 µL donde se depositaron entre 100-150 COCs. Las gotas fueron cubiertas con aceite mineral y la maduración se efectuó en una atmósfera de 5% de CO_2 en aire, a 39º C de temperatura y con una humedad relativa del 95-100% durante 44 h. El medio de maduración fue suplementado con hormonas las primeras 22 h mientras que no hubo suplementación hormonal las 22 h restantes.

Fecundación *in vitro*

La fecundación se realizó en medio TBMm suplementado según el diseño experimental de cada trabajo. Después de la maduración, los COCs fueron denudados con 0'1% de hialuronidasa en medio de maduración mediante agitación con un vortex durante 2 min, a 1.660 vueltas/min. Los ovocitos fueron lavados dos veces en medio de maduración y 3 veces en medio de fecundación previamente equilibrado en el incubador. Grupos de 30 - 50 ovocitos denudados, dependiendo del experimento, fueron introducidos en microgotas de 50 µL de medio de fecundación en una placa Petri de 35 x 10 mm (Falcon, Becton Dickinson Labware, Franklin Lakes, USA). Las microgotas fueron cubiertas con aceite mineral y mantenidas en el incubador (5% de CO_2 en aire, 39º C), durante aproximadamente 30 min hasta la adición de los espermatozoides.

Criopreservación del semen

El semen fue procesado para su congelación por un procedimiento modificado por Roca y colaboradores (2003) basado en el método originalmente descrito por Westendorf y colaboradores (1975), con las modificaciones señaladas por Maxwell y Johnson (1997) y Thurston y colaboradores (1999). Brevemente, la fracción rica del eyaculado recogida por el método manual, se diluyó inmediatamente 1:1 en diluyente BTS (Beltsville Thawing Solution; Pursel y Johnson, 1975) a la misma temperatura (30-35°C) y se llevo a una vitrina térmica en la cual se realizó un descenso de temperatura suave y paulatino durante aproximadamente 3 h hasta 17ºC. Posteriormente, a esta temperatura las muestras espermáticas se centrifugaron (Megafuge 1.0 R, Heraeus, Alemania) a 2.400 x g durante 3 minutos (Carvajal et al., 2004) con el fin de eliminar el sobrenadante. Los pellets resultantes se diluyeron en diluyente Lactosa-Yema de huevo (LEY; Westendorf y cols., 1975) hasta alcanzar una concentración de 1'5 x 10^9 espermatozoides/mL. Una vez diluido el semen en este medio, se realizó un nuevo descenso de temperatura suave y paulatino en la vitrina térmica durante aproximadamente 90 -120 min hasta los 5ºC. Una vez alcanzada esta temperatura, se rediluyó hasta conseguir una concentración final de 1 x 10^9 espermatozoides/mL con el diluyente LEY-Glicerol-Orvus Es Paste (LEYGO; Westendorf y cols., 1975). Inmediatamente después y dentro de la vitrina térmica a 5°C, las muestras seminales diluidas fueron envasadas en pajuelas de plástico de 0'5 mL (Minitüb, Alemania), selladas con una selladora automática y congeladas bajo condiciones controladas usando un biocongelador (IceCube 1810, Minitüb Alemania) con las siguientes velocidades de congelación: de 5ºC a -5ºC a 6ºC/min, de -5ºC a -80ºC a 40ºC/min,

mantenidas durante 30 segundos a -80ºC y posteriormente enfriadas a 70ºC/min hasta alcanzar los -150ºC. Una vez envasadas y congeladas las muestras espermáticas se almacenaron en tanques de nitrógeno líquido hasta el momento de su utilización.

Descongelación del semen

Las pajuelas de semen se descongelaron en un baño de agua a 37ºC durante 20 segundos. Después de la descongelación, la evaluación espermática se realizó a los 30 min usando un pool de 2 pajuelas procedentes de cada verraco. La motilidad se analizó con un sistema CASA de análisis objetivo de la motilidad (Sperm Class Analyzer®, SCA, Microptic, Barcelona, Spain). Tanto la motilidad espermática como las anormalidades acrosómicas se analizaron para cada replicado de cada experimento realizado.

Preparación del semen para la FIV, fecundación y cocultivo de los gametos

Antes de proceder a la fecundación *in vitro*, el semen descongelado (150 µL) se lavó 3 veces en 10 ml de medio PBSDm mediante centrifugación a 1.900 g durante 3 min. El pellet resultante fue resuspendido en medio de fecundación y después de una apropiada dilución, según la requerida en cada experimento, 50 µL de cada suspensión espermática fueron añadidos a los 50 µL del medio que contenían a los ovocitos. Los gametos fueron coincubados bajo una atmósfera de 5% de CO_2, a 39ºC y con una humedad del 95-100% durante diferentes tiempos de coincubación requeridos según el experimento.

Cultivo *in vitro*

Tras el periodo de coincubación de los gametos requerido en cada experiencia, los zigotos fueron retirados del medio de fecundación y lavados 3 veces en medio de cultivo previamente equilibrado. Posteriormente, los zigotos fueron transferidos a una placa de 4 pocillos (30 - 50 zigotos por pocillo según el experimento, en un volumen de 500 µL del mismo medio cubierto con aceite mineral) y cultivados bajo una atmósfera de 5% CO_2 en aire, a 39ºC durante 12-15 h para analizar los parámetros de fecundación y 7 días con el fin de evaluar el desarrollo embrionario hasta el estadio de blastocisto.

Análisis de la maduración, penetración espermática y desarrollo embrionario

Con el fin de evaluar la maduración y los parámetros de fecundación, los ovocitos y los zigotos se montaron en portaobjetos y se fijaron en una solución de acético:etanol (1:3 v:v) a temperatura ambiente durante 48-72 h. Posteriormente, los zigotos fueron teñidos con 1% lacmoid en 45% (v/v) de ácido acético y examinados bajo un microscopio de contraste de fase a 20 y a 40 aumentos. La tasa de maduración se analizó a las 44 h de la MIV. Los ovocitos con cromosomas en metafase-II y con un cuerpo polar extruído fueron considerados como ovocitos maduros. Los parámetros de FIV se evaluaron a las 15 h después de la inseminación. Los ovocitos fueron considerados como penetrados cuando tenían al menos una cabeza de espermatozoide descondensada y/o un pronúcleo masculino, estando presentes su correspondiente flagelo y los dos cuerpos polares. Los ovocitos degenerados, inmaduros u ovocitos con el oolema roto o con una apariencia anormal del citoplasma fueron excluidos de los experimentos. Los parámetros de fecundación evaluados fueron:

Penetración: porcentaje de ovocitos penetrados por uno o más espermatozoides con respecto al total de ovocitos maduros.

Monospermia: porcentaje de ovocitos conteniendo sólo una cabeza de espermatozoide o un pronúcleo masculino y los dos cuerpos polares con respecto al total de los ovocitos penetrados.

Número de espermatozoides por ovocito: número de espermatozoides, en cualquier estadio de descondensación por ovocito penetrado.

Eficiencia de la fecundación: porcentaje de ovocitos monospérmicos en relación al total de ovocitos inseminados.

En determinados experimentos se analizó el número de espermatozoides adheridos a la zona pelúcida. Para ello, los ovocitos procedentes de cada grupo de coincubación según el diseño de cada experimento se lavaron en medio DPBS y se montaron en portas en una dilución de 4 µl de Glicerol- DPBS (3:1) con 10 µg/ml de Hoechst (Hoechst-33342). El número de espermatozoides adheridos a la zona pelúcida fue evaluado usando un microscopio de fluorescencia a 40 aumentos.

El desarrollo embrionario se evaluó a los dos días, con el fin de observar las divisiones, entendiendo como divididos aquellos embriones en estadio de 2-4 células y a los 7 días con el objetivo de examinar aquellos embriones que alcanzaron el estadio

de blastocisto. Tanto las divisiones embrionarias (embriones en estadio de 2-4 células/total de cultivados) como la formación de blastocistos (número de blastocistos/total de cultivados) se evaluaron mediante un estereomicroscopio. El número total de células, como un indicador de la calidad embrionaria, se analizó mediante montaje de cada blastocisto en portaobjetos en gotas de 4 µL de una solución de Glicerol-DPBS (3:1) con 10 µg/mL de Hoechst- 33342 y posteriormente se procedió al recuento de las células mediante visualización en un microscopio de fluorescencia.

DISEÑO EXPERIMENTAL

Objetivo 1: Evaluar el efecto de diferentes ratios espermatozoide:ovocito durante la FIV utilizando un tiempo de coincubación corto (10 min) y diferentes periodos de post-coincubación en un medio no apropiado para la FIV sobre los parámetros de fecundación (Artículo 1).

Con el fin de alcanzar el primer objetivo se realizaron dos experimentos. En el primero de ellos, (experimento 1.1), se utilizó un total de 1.624 ovocitos madurados *in vitro* en cuatro replicados, que se inseminaron con espermatozoides congelados-descongelados a diferentes ratios de espermatozoide:ovocito (2.000, 1.500, 1.000 y 500 espermatozoide por ovocito ó 12 x 10^5, 9 x 10^5, 6 x 10^5 y 3 x 10^5 espermatozoides/ml, respectivamente) y se coincubaron durante 10 min ó 6 h, en un diseño experimental factorial 4 x 2. Los ovocitos coincubados durante 10 min fueron lavados mediante pipeteo mecánico tres veces en medio limpio de FIV con el fin de eliminar los espermatozoides no adheridos a la zona pelúcida, y transferidos a otra gota e incubados en medio FIV (sin espermatozoides) durante 6 h. Transcurridas estas 6 h, los zigotos fueron lavados y transferidos a medio de cultivo de embriones durante 12-15 h y posteriormente, fueron fijados y teñidos para evaluar los parámetros de fecundación. En el segundo experimento (experimento 1.2), se utilizó un total de 1.872 ovocitos madurados *in vitro* procedentes de tres replicados, los cuales se inseminaron con los mismos ratios que el experimento anterior y se coincubaron durante 10 min, con el consiguiente lavado una vez finalizado este corto periodo de coincubación. Posteriormente, se transfirieron a tres medios distintos de post-coincubación tal y como se indica a continuación:

(1) Directamente al medio de cultivo *in vitro* (CIV) durante 18 h (grupo A);

(2) Primero al medio limpio de FIV (sin espermatozoides) solamente por un periodo de 2 h y entonces lavados y transferidos al medio de CIV durante 16 h (grupo B);

(3) En primer lugar al medio limpio de FIV (sin espermatozoides) coincubados durante 6 h, lavados y transferidos al medio de CIV durante 12 h (grupo C, control).

Al finalizar los diferentes periodos de CIV, los zigotos se tiñeron y se fijaron con el fin de evaluar los parámetros de fecundación.

***Objetivo* 2:** Optimizar un protocolo de FIV para machos individuales, empleando como aditivos la cafeína, el ácido hialurónico y la adenosina en el medio de fecundación y diferentes tiempos de coincubación de los gametos (Artículo 2).

En primer lugar, se estudió el efecto de la cafeína, el AH y la adenosina durante la coincubación de los gametos sobre los parámetros de fecundación (experimento 2.1). Para ello se utilizaron un total de 2.042 ovocitos madurados *in vitro* en tres replicados y semen procedente de dos verracos (A y B), (1.055 ovocitos para el verraco A y 987 ovocitos para el verraco B). Las concentraciones utilizadas de cada aditivo en el medio de FIV fueron las siguientes: de cafeína 2 mM, de AH 0'5 mg/mL (Bayer, Hyonate vet, Barcelona, España) y de adenosina 10 µM. Cada aditivo a estudiar se añadió a un medio de FIV, solo o en combinación, de manera que se prepararon un total de ocho medios distintos de FIV:

(1) mTBM + cafeína
(2) mTBM + cafeína + AH
(3) mTBM + cafeína + adenosina
(4) mTBM + cafeína + AH + adenosina
(5) mTBM
(6) mTBM + AH
(7) mTBM + adenosina
(8) mTBM + AH + adenosina

En segundo lugar, se evaluó el efecto de las diferentes concentraciones de AH y adenosina durante la FIV (experimento 2.2). Las concentraciones utilizadas para cada aditivo en este caso fueron: para el AH 0, 0'5, 1'0 y 1'5 mg/mL y para la adenosina 0, 10,

20 y 40 µM. Este experimento se diseñó según las necesidades de cada macho basándose en los resultados del experimento anterior. De esta forma, los espermatozoides del verraco A fueron lavados e introducidos en medio de FIV con AH o adenosina, pero en ausencia de cafeína. Por el contrario, los espermatozoides del verraco B fueron lavados e introducidos en medio de FIV con AH o adenosina, en presencia de cafeína. En este experimento se utilizaron un total de 1.961 ovocitos madurados *in vitro* en tres replicados (965 para el verraco A y 996 para el verraco B) que fueron inseminados, fijados y teñidos con el fin de analizar los parámetros de fecundación. Por último, en un tercer experimento (experimento 2.3) se evaluó el efecto del tiempo de coincubación de los gametos sobre los parámetros de fecundación. Para ello, se utilizaron dos periodos de coincubación de los gametos, 10 min y 6 h, en cada uno de los verracos anteriormente analizados. Además, teniendo en cuenta los resultados de los dos experimentos anteriores, los espermatozoides fueron lavados y coincubados con los ovocitos durante 10 min y 6 h en medio de FIV sin aditivos para el verraco A y en presencia de cafeína únicamente para el verraco B. Los ovocitos coincubados durante 10 min fueron lavados como ya se ha mencionado anteriormente. En este experimento se utilizaron un total de 1.940 ovocitos madurados *in vitro* en tres replicados distintos (945 para el verraco A y 995 para el verraco B).

Objetivo 3: Examinar el efecto de tiempos de coincubación ultracortos sobre la eficiencia de la fecundación *in vitro* porcina (Artículo 3).

En este estudio (experimento 3.1) se utilizó un total de 2.833 ovocitos madurados *in vitro* procedentes de tres replicados distintos que se inseminaron con espermatozoides congelados-descongelados y se coincubaron durante 0’25, 1, 2, 3, 7, 10 min y 6 h. Los ovocitos pertenecientes a los periodos de coincubación desde 0’25 min hasta 10 min se lavaron tres veces en medio limpio de fecundación usando una pipeta adelgazada como se ha indicado en anteriormente y posteriormente se cultivaron en medio de fecundación (sin espermatozoides) hasta completar las 6 h de coincubación. En este experimento se evaluaron tanto los parámetros de FIV como los espermatozoides adheridos la zona pelúcida.

Objetivo 4: Estudiar si la combinación de un sistema de FIV en pajuela con un tiempo de de coincubación corto podría ser una estrategia adecuada para disminuir la polispermia y mejorar la producción de blastocistos porcinos *in vitro* (Artículo 4).

En un primer estudio (experimento 4.1) se utilizaron 723 ovocitos madurados *in vitro* en tres replicados con el fin de evaluar tres sistemas de FIV distintos: sistema FIV en pajuela y 10 min de coincubación, sistema de FIV en pajuela y 6 h de coincubación y sistema de FIV en microgota con 6 h de coincubación sobre los parámetros de FIV. La fecundación en pajuela se realizó utilizando varios ratios de espermatozoide:ovocito (20.000, 30.000, 40.000, y 50.000). En el sistema tradicional de la microgota, se utilizó un ratio de 1.000 espermatozoides:ovocito. Los ovocitos coincubados en el interior de la pajuela durante 10 min fueron extraídos de ésta y posteriormente lavados tal y como se ha indicado en anteriores experimentos. En un segundo experimento (experimento 4.2) se utilizaron 862 ovocitos madurados *in vitro* en un total de tres replicados que se fecundaron mediante dos sistemas: el sistema de FIV en pajuela y 10 min de coincubación y el sistema de microgota, con el objetivo de comparar el desarrollo embrionario hasta el estadio de blastocisto y el número total de células por blastocisto en ambos sistemas. Los ovocitos fueron inseminados con 20.000 espermatozoides:ovocito en el sistema en pajuela y 1.000 espermatozoides:ovocito en la microgota.

Objetivo 5: Determinar si el efecto de la administración de dos metabolitos del retinol, 9- *cis* ácido retinoico y all- *trans* retinol, a diferentes concentraciones durante la maduración *in vitro* sobre los parámetros de FIV y el desarrollo embrionario (Artículo 5).

En un primer experimento (experimento 5.1) se evaluó el efecto del 9- *cis* ácido retinoico a diferentes concentraciones (0, 5, 50 y 500 nM) durante la MIV de los ovocitos sobre la maduración, los parámetros de FIV y sobre el desarrollo embrionario. Para ello se utilizó un total de 2.161 ovocitos madurados *in vitro* procedentes de cinco replicados distintos. En un segundo experimento (experimento 5.2), se estudió el efecto del all- *trans* retinol a diferentes concentraciones (0, 125, 1.250 y 12.500 nM) durante la MIV sobre los mismos parámetros anteriormente mencionados, utilizando un total de 2.141 ovocitos madurados *in vitro* en cinco replicados.

ANÁLISIS ESTADÍSTICO

Todos los datos obtenidos se analizaron con el paquete estadístico SPSS, versión 13.0 (SPSS Inc., Chicago, III). Para el análisis estadístico se utilizó el modelo lineal mixto de análisis de varianza (ANOVA), variando los efectos fijos y aleatorios según los factores estudiados en cada experimento. Los porcentajes de penetración, monospermia, los datos de eficiencia de la fecundación *in vitro* y la formación de blastocistos fueron modelados según el modelo binomial de los parámetros descritos por Fisz (Fisz, 1980) antes del análisis. Cuando el análisis de varianza mostró un efecto significativo los valores fueron comparados usando el Test de Bonferroni. El umbral de significancia fue ajustado a $p<0'05$. Los resultados se expresan como medias ± SEM.

RESULTADOS

Objetivo 1: Evaluar el efecto de diferentes ratios espermatozoide:ovocito durante la FIV utilizando un tiempo de coincubación corto (10 min) y diferentes periodos de post-coincubación en un medio no apropiado para la FIV sobre los parámetros de fecundación (Artículo 1).

En el primer experimento (experimento 1.1) se observó un efecto significativo del ratio espermatozoide:ovocito en todos los parámetros de fecundación estudiados. La eficiencia de la FIV (número de ovocitos monospérmicos/total de ovocitos inseminados) fue mayor ($p<0'05$) en los ovocitos coincubados con espermatozoides durante 10 minutos e inseminados con 1.500 y 1.000 espermatozoides:ovocito (35'8±3'7 - 37'6±2'7%, respectivamente) y en los coincubados durante 6 horas con 500 espermatozoides por ovocito (37'2±3'1%). En el segundo experimento (experimento 1.2), los porcentajes de penetración y de eficiencia de la FIV obtenidos en el grupo A, cuando los zigotos fueron transferidos directamente a medio CIV, fueron muy pobres (entre 3 y 15%) independientemente del ratio espermatozoides:ovocito. Sin embargo, en el grupo B, cuando se transfirieron durante dos horas al medio de FIV, los parámetros de fecundación obtenidos fueron similares a los del grupo control y se observó un efecto claro del ratio espermatozoides:ovocito. Estos resultados demuestran que el tiempo de coincubación se puede reducir a 10 minutos como estrategia para incrementar la eficiencia de la fecundación dependiendo del ratio espermatozoides:ovocito utilizado, y que los

espermatozoides unidos a la zona pelúcida requieren un máximo de 2 h en un medio apropiado para penetrar a los ovocitos *in vitro*.

***Objetivo 2**:* Optimizar un protocolo de FIV para machos individuales, usando como aditivos la cafeína, el ácido hialurónico y la adenosina en el medio de fecundación y diferentes tiempos de coincubación de los gametos (Artículo 2).

Los resultados del experimento 2.1, indicaron que sólo la presencia o ausencia de cafeína en el medio de FIV tuvo un efecto significativo en la eficiencia de la fecundación, observándose una clara influencia del verraco. Mientras que para el verraco B la adición de cafeína fue necesaria para alcanzar aceptables tasas de penetración y monospermia, la presencia de cafeína redujo considerablemente las tasas de monospermia en el verraco A. En el experimento 2.2, se observó un aumento de la penetración en el verraco A al aumentar las concentraciones de AH añadidas al medio, sin afectar los demás parámetros de fecundación. Sin embargo, al probar las mismas concentraciones de AH en el verraco B, no se observaron diferencias en los parámetros de FIV. Por otra parte, la adición de las diferentes concentraciones de adenosina al medio de FIV no influyó en ninguno de los parámetros de FIV en ninguno de los dos verracos. Los resultados del experimento 2.3 demostraron que la utilización de un tiempo de coincubación corto (10 min) aumentó significativamente ($p < 0'001$) la tasa de penetración y el número de espermatozoides por ovocito en el verraco A, pero se redujo la monospermia. Sin embargo en el verraco B, el tiempo de coincubación no afectó la eficiencia de la fecundación. A la vista de los resultados del presente estudio, podemos concluir que son necesarios estudios preliminares para cada verraco con el fin de seleccionar las condiciones óptimas para la FIV.

***Objetivo 3**:* Examinar el efecto de tiempos de coincubación ultracortos sobre la eficiencia de la fecundación *in vitro* porcina (Artículo 3).

Los resultados del presente experimento (experimento 3.1) muestran como el número de espermatozoides unido a la zona pelúcida aumentó significativamente ($p<0'02$) a medida que aumentó el tiempo de coincubación (8'0±2'8, 10'8±2'.8, 13'2±2'8, 20'9±2'8, 31'6±2'8, 32'3±2'8 y 44'2±2'8, para 0'25, 1, 2, 3, 7, 10 min y 6 h, respectivamente), aunque no se observó ningún incremento en la tasa de penetración en los grupos de 2 min hasta las 6 h (valores desde 53'5±2'8 a 61'3±2'6%). La reducción

del tiempo de coincubación no afectó a la tasa de monospermia, con valores entre 71'3±3'4 y 80'2±3'8, ni al número de espermatozoides por ovocito penetrado (valores desde: 1'2±0'4 a 1'4± 0'5). A la vista de los resultados del presente estudio, podemos concluir que bajo condiciones *in vitro* se puede obtener una alta tasa de penetración utilizando tiempos de coincubación tan cortos como 2 min, aunque la monospermia no pudo ser mejorada mediante esta estrategia.

Objetivo 4: Estudiar si la combinación de un sistema de FIV en pajuela con un tiempo de de coincubación corto podría ser una estrategia adecuada para disminuir la polispermia y mejorar la producción de blastocistos porcinos *in vitro* (Artículo 4).

En el experimento 4.1, cuando se utilizaron los dos sistemas de FIV en pajuela con 10 min y 6 h de coincubación, se observó que el sistema de FIV en pajuela con 10 min de coincubación aumentó significativamente ($p<0'001$) la tasa de penetración y la eficiencia de la de la FIV (67'7±6'4% vs. 31'9±6'5% y 41'5±2'5% vs. 17'6±2'5%, para 10 min y 6 h respectivamente), mientras que no se observaron diferencias significativas en la incidencia de monospermia entre los dos sistemas (64'3±5'1 y 67'7±3'4%, para 10 min y 6 h, respectivamente). La tasa de penetración en el sistema de FIV en la microgota y con 6 h de coincubación fue mayor (93'8±3'65; $p<0'001$) que la del sistema de FIV en pajuela y 10 min de coincubación (67'7±6'4%), sin embargo, la monospermia fue severamente reducida en el sistema de microgota (25'0±4'3% vs. 67'7±3'4%, para el sistema de FIV en microgota y 6 h de coincubación y el sistema de FIV en pajuela y 10 min de coincubación, respectivamente). En cuanto a la eficiencia de la FIV se observaron unos valores similares entre el sistema de microgota y el de pajuela con 6 h de coincubación, pero la eficiencia fue significativamente mayor ($p<0'05$) cuando se utilizó el sistema de FIV en pajuela y 10 min de coincubación. En el experimento 4.2, se comparó la PIV de embriones en dos sistemas: el sistema de FIV en pajuela con 10 min de coincubación y un ratio de 30.000 espermatozoides:ovocito (basándonos en los mejores resultados del experimento anterior) y el sistema de FIV en microgota y 6 h de coincubación con 1.000 espermatozoides:ovocito. La tasa de formación de blastocisto mostró una tendencia ($p = 0'06$) a ser más elevada cuando se utilizó el sistema de FIV con 10 min comparado con el sistema de FIV en microgota y 6 h de coincubación. Además, el número total de células/blastocisto aumentó significantemente ($p<0'05$) con el sistema de FIV en pajuela con 10 min de coincubación. Estos resultados muestran que el sistema de FIV en pajuela con 10 min de coincubación es una estrategia efectiva para

disminuir la penetración polispérmica, y mejorar la eficiencia de la FIV y la calidad de los blastocistos en términos de número total de células por blastocisto.

Objetivo 5: Determinar el efecto de la administración de dos metabolitos del retinol, 9- *cis* ácido retinoico (RA) y all- *trans* retinol (ROH), a diferentes concentraciones durante la maduración *in vitro* sobre los parámetros de FIV y el desarrollo embrionario (Artículo 5).

En el experimento 5.1, se evaluó el efecto del RA durante la MIV. Se observó que las tasas de maduración de los ovocitos disminuyeron significativamente ($p<0'001$), con valores por debajo del 5%, cuando se utilizó la concentración más alta de este retinoide (500nM), mientras 5 y 50 nM de RA no tuvieron ningún efecto comparado con el control. La concentración de 5 nM de RA mejoró la tasa de desarrollo embrionario hasta el estadio de blastocisto ($p<0'001$). En el experimento 5.2, al evaluar el efecto del ROH durante la MIV, no se observaron diferencias en la tasa de maduración de los ovocitos entre 125 nM, 1.250nM de ROH y el control, pero el uso de 12.500 nM de ROH fue perjudicial. La tasa de penetración fue menor al utilizar la concentración de 1.250nM que la de 125nM de ROH o el control, sin embargo la formación de blastocistos no fue diferente entre los tres tratamientos de maduración. Por lo tanto, 5 nM de RA en el medio de MIV aumentó significantemente el porcentaje de blastocistos, sugiriendo que el RA puede jugar un importante papel durante la MIV.

CONCLUSIONES

1. El tiempo de coincubación puede ser reducido a 10 minutos como estrategia para incrementar la eficiencia de la fecundación dependiendo del ratio espermatozoides:ovocito utilizado. La post-incubación de los ovocitos con los espermatozoides unidos a la zona pelúcida en un medio no apropiado para la FIV no mejora la eficiencia de la fecundación. Los espermatozoides unidos a la zona pelúcida requieren un máximo de 2 h en medio apropiado para penetrar los ovocitos (Artículo 1).

2. Las necesidades de los espermatozoides para la FIV en términos de aditivos del medio de fecundación y tiempo de coincubación varían entre verracos (Artículo 2).

3. Un tiempo de coincubación tan corto como 2 min es suficiente para obtener unas tasas de penetración, monospermia y una eficiencia de la FIV similares a las alcanzadas por los sistemas de FIV porcina actuales donde utilizan tiempos largos (6 h) de exposición (Artículo 3).

4. El sistema de FIV en pajuela junto con un corto tiempo de coincubación (10 min) aumenta la penetración monospérmica, mejora la eficiencia de la FIV y la calidad de los blastocistos en términos de número de células por blastocisto comparado con el sistema tradicional en microgota y 6 h de coincubación. Además este sistema permite el uso de un amplio rango de ratios espermatozoide:ovocito (Artículo 4).

5. La adición de 5 nM de *9- cis* ácido retinoico al medio de MIV aunque no mejora la tasa de maduración ni de fecundación en comparación con el grupo control, aumenta significativamente la formación de blastocistos (Artículo 5).

Extended Summary

Extended Summary 9

INTRODUCTION

During the last few years, important advances in reproductive technologies have been made for medical purposes and for optimizing animal breeding. It is worth highlighting the growing interest in biotechnologies such as transgenesis, animal cloning and the generation of embryonic stem cells. Those technologies require in vitro production of large quantities of matured oocytes and embryos, thus there is an increasing reliance on the *in vitro* fertilization laboratories to maximize embryo viability and quality. Swine are increasingly used in transgenic technologies, including gene manipulation and nuclear transfer and has been considered as an animal model for human diseases due the immense potential that present for xenotransplantation. These facts have reaffirmed the need for a more efficient *in vitro* embryo production system for pigs.

Although substantial progress has been made in *in vitro* culture, fertilization and maturation (IVC, IVF and IVM) of porcine oocytes, the overall efficiency of *in vitro* production of porcine embryos has been limited by low rates of development to the blastocyst stage (Abeydeera and Day, 1997a; Kikuchi et al., 2002) and their poor quality in comparison with blastocyst produced i*n vivo* (Wang et al., 1999). The low quality of oocytes after *in vitro* maturation (Funahashi et al., 1997; Nagai, 2000; Kikuchi et al., 2002), increased polyspermy after IVF (Niwa, 1993; Abeydeera and Day, 1997a; Wang et al., 1997; Abeydeera, 2002; Gil et al., 2004a, b, 2007; Almiñana et al., 2005, 2007a, b) and poor developmental ability and quality of embryos produced by IVM-IVF (Abeydeera and Day, 1997a; Wang et al., 1999; Han et al., 1999a, b; Abeydeera, 2001; McCauley et al., 2003) are the main reasons for this limited performance, together with the unsuitability of *in vitro* culture systems (Kikuchi et al., 2002).

In spite of all attempts reported in the literature, the incidence of polyspermic penetration in porcine oocytes fertilized *in vitro* is still one of the major obstacles in porcine IVF systems and remains extremely high compared with that in other species (Niwa, 1993; Funahashi and Day, 1997; Prather and Day, 1998; Day, 2000; Funahashi, 2003). This abnormality could be due to inadequate and delayed establishment of the in vitro matured zona block and/or the conditions involved during IVF (revised by Abeydeera, 2002). Conditions such as the coculture medium (Fraser, 1995; Abeydeera and Day, 1997b; Martinez-Madrid et al., 2001; Kidson et al., 2001), sperm:oocyte ratio (Rath, 1992; Xu et al., 1996; Gil et al., 2004a), source of spermatozoa (Wang et al., 1994; Abeydeera and Day, 1997a; Gil et al., 2005), the IVF system (Funahashi and Nagai, 2000; Beebe et al., 2002; Li et al., 2003; Clark et al., 2002, 2003, 2005) and coincubation time (Coy et al., 1993; Oocampo et al., 1994; Abeydeera and Day, 1997a; Marchal et al., 2002; Gil et al., 2004b) affect the sperm penetration and subsequently the polyspermy rate.

In this regard, a reduction of the period of sperm–oocyte coincubation during porcine IVF has also been investigated as an alternative method to reduce polyspermic fertilization and to improve IVF efficiency (Cheng et al., 1986; Mattioli et al., 1989; Abeydeera and Day, 1997a). Abeeydera and Day (1997a) reported that 3 h after IVF, 31% of oocytes were penetrated, with maximum penetration attained by 6 h and without any further increase at 9 or 12 h of coincubation. On the contrary, such extension of sperm–oocyte coincubation to 6–12 h resulted in a significant increase in polyspermy rate. Currently, the majority of laboratories working on porcine IVF have reduced the time of gamete coincubation from the 12–18 h used in earlier porcine IVF systems (Cheng et al., 1986; Mattioli et al., 1989), to 5–6 h (Abeydeera and Day, 1997a; Suzuki et al., 2000; Gil et al., 2003). Recently, a new strategy has been proposed to improve the efficiency of *in vitro* pig embryo production by minimizing the number of spermatozoa present in the fertilization medium (Grupen and Nottle, 2000). However, this short time of co-exposure of the gametes has lead to contradictory results among laboratories (Grupen and Nottle, 2000; Gil et al., 2004b). All short coincubation times reported in previous studies shared the common practice of washing the oocytes by mechanical pipetting in fresh IVF medium to remove spermatozoa not bound to the zona, transferring the washed oocytes to another droplet of IVF medium (containing no spermatozoa) and incubating them until 5–6 h of a normal IVF coincubation time are completed. It has been shown that spermatozoa bound to the zona within the first 10 min are able to fertilize a high number of oocytes (Grupen and Nottle, 2000; Gil et al., 2004b; Funahashi and Romar, 2004; Almiñana et al., 2005). Moreover, it is known that sperm penetration of *in*

vitro matured porcine oocytes occurs as early as 2 h post-insemination (Hunter and Dziuk, 1968; Marchal et al., 2002).Therefore, a possible way to increase the efficiency *in vitro* of short coincubation times could be to use post-coincubation periods in media that are not suitable for IVF. On the other hand, it would be necessary to set the suitable sperm:oocyte ratio for IVF systems, since it can be presumed that the effectiveness of the use of short periods of coincubation during IVF to decrease polyspermy depends on the sperm:oocyte ratio. Thus, the first objective was to evaluate the effect of different sperm:oocyte ratios during IVF, using a short coincubation time (10 min), and different periods of post-coincubation in a medium not appropriate for IVF, on *in vitro* fertilization parameters.

Addition of different substances to IVF medium has been studied in attempt to decrease the incidence of polyspermy during in vitro fertilization. In most current porcine IVF systems (Yoshida, 1987; Nagai et al., 1988; Mattioli et al., 1989; Wang et al., 1991; Funahashi and Day, 1993) boar spermatozoa are exposed to caffeine, which is known to enhance sperm motility (Garbers et al., 1973) and, presumably, to stimulate capacitation (Wang et al., 1991; Funahashi et al., 2000a) and spontaneous acrosome reactions (ARs) (Funahashi et al., 2000b). However, it has been suggested that the ARs induced by caffeine may be related to the high incidence of polyspermic fertilization (Funahashi and Nagai, 2001). Recently, polyspermic penetration has been greatly reduced by replacing caffeine with adenosine in fertilization medium (Funahashi et al., 2000a, b; Funahashi and Nagai, 2001). In those studies, the analysis of the functional state of spermatozoa exposed to this additive revealed that adenosine stimulated capacitation but inhibited spontaneous ARs. Similar to adenosine, hyaluronic acid (HA) appears to induce sperm capacitation without the subsequent ARs seen in its absence (Rodriguez- Martinez et al., 1998). Furthermore, since HA has been localized in the intraluminal fluid of the porcine oviduct (Tienthai et al., 2001) and it is secreted by porcine cumulus oocyte complexes (COCs) during IVM (Yokoo et al., 2002; Suzuki et al., 2002), it may play a role during IVF and polyspermy. In spite of the above mentioned studies, only a few, contradictory experiments have been performed in vitro to evaluate the effect of HA on polyspermic fertilization (Suzuki et al., 2000; Suzuki et al., 2002) and, to the best of our knowledge, no study has reported the combinatorial effect of these supplementations during gamete coincubation on polyspermic fertilization. On the other hand, it is widely accepted that penetration and polyspermy rates in pig IVF are also affected by large variations among individual males when fresh or frozen/thawed semen is used (Wang et al., 1991; Sirard et al., 1993; Suzuki et al., 1994; Wang et al., 1995). These considerable differences among boars suggest that all males do not respond to IVF conditions equally. Therefore, defining

the best *in vitro* conditions for spermatozoa from each individual male is obviously desirable to achieve an optimal IVF system that can provide reproducibly-low rates of polyspermy. The second objective was to increase the efficiency of porcine IVF by optimizing an IVF protocol for individual males. Differences between boars were evaluated in response of their spermatozoa to IVF using various additives for the fertilization medium and different intervals of gamete co-incubation. Taking in account that coincubation time is an important factor during IVF, different periods of gamete coincubation were used in this study.

In most current IVF systems, oocytes are exposed to an excessive and nonphysiologically high number of spermatozoa for a long period. However, it has been reported that *in vitro* penetration of porcine oocytes as well as polyspermic fertilization occur as early as 2–3 h postinsemination with frozen–thawed spermatozoa (Abeydeera and Day, 1997a; Marchal et al., 2002; Gil et al., 2007) or with fresh semen (Martinez et al., 1996). Moreover, it is also known that the percentage of true acrosome reacted sperm remained reasonably constant throughout coincubation (Vazquez et al., 1993). This suggests that extending spermatozoa–oocyte coincubation times tends to increase spermatozoa–oocyte interactions resulting in a high incidence of polyspermic penetration. Therefore, it seems obvious that a long exposure of oocytes to spermatozoa may be not necessary or may even be harmful. Our previous results (Gil et al., 2004; Almiñana et al., 2005; Gil et al., 2007) together with the fact that good fertilization rates and a low incidence of polyspermy can be obtained using ultrashort (30 s) coincubation (Bungum et al., 2006) in human IVF, suggest that 10 min of gamete coincubation may still be a period too long. Thus, the third objective was to evaluate the effect of ultrashort coincubation times on the efficiency of porcine IVF.

As mentioned above, although the IVF conditions differ among laboratories, a common factor in current IVF systems is the exposition of oocytes to an excessive and non-physiological number of sperm during gamete coincubation. This high number of spermatozoa per oocyte causes simultaneous sperm penetration and results in polyspermic fertilization (Wang et al., 2003). In addition, the environment conditions during *in vitro* gamete coincubation are far from the oviductal environment in the female genital tract. These IVF conditions appear to override the *in vivo* strategies for sperm capacitation and natural selection of spermatozoa after insemination (Hunter and Rodriguez-Martinez, 2004). Thus, a reduction in spermatozoa number during IVF has been studied in order to decrease polyspermic penetration, however, it also reduces overall sperm penetration rates (Abeydeera and Day, 1997a) resulting in low efficiency of

fertilization. New strategies such as the straw-IVF system (Li et al., 2003), the "climbing-over- wall" method (Funahashi and Nagai, 2000), or microfluidic technology (Beebe et al., 2002; Clark et al., 2002, 2003, 2005) have been reported in an attempt to mimic the process of in vivo fertilization in the oviductal tract, where spermatozoa are gradually capacitated during transport to the site of fertilization, (Rodriguez-Martinez et al., 2005), reducing the incidence of polyspermic fertilization (Funahashi and Nagai, 2000). In attempt to minimize a massive exposition of spermatozoa to oocytes, an alternative IVF system to traditional microdrop-IVF system in combination with a short coincubation time was proposed. Since, it may be possible that the combination of strategies and not the study of an isolated factor, could improve the yield of IVF. Based on all the data above mentioned, the fourth objective was to evaluate whether the combination of a straw-IVF system and a short coincubation time (10 min) is a suitable strategy to decrease polyspermic fertilization and improve *in vitro* production of porcine blastocysts.

Together with the gamete coincubation conditions, *in vitro* maturation seems a key factor determining IVF results. Moreover, embryo development is strongly influenced by the oocyte maturation conditions. However, although successful nuclear maturation of oocytes *in vitro* is obtained with a high level of repeatability in most current porcine IVF systems, the IVM environment may not support adequate cytoplasmic and molecular maturation (Sirard et al., 2006). This cytoplasmic and molecular maturation is required to prepare the oocyte for post-fertilization events, allowing the oocyte to reach the blastocyst stage. Compared to *in vivo* maturation, IVM conditions are simple and materially limited, which can profoundly affect the maturation status of oocyte. For this reason, improved culture conditions will be essential to obtain consistently successful and reliable oocyte maturation (both cytoplasmic and nuclear), leading to dramatically improved efficiency of *in vitro* embryo production. The beneficial effects of *9- cis* retinoic acid (RA) and *all-trans* retinol (ROH) have been demonstrated in bovine oocytes, especially on cytoplasmic competence after *in vitro* maturation (Duque et al., 2002; Gomez et al., 2003; Ikeda et al., unpublished data) and embryonic development and quality (Duque et al., 2002; Hidalgo et al., 2003; Gomez et al., 2003; Livingston et al., 2004; Lima et al., 2006). Besides, there is growing evidence of the essential role of retinol and its metabolites in cell growth, differentiation and embryonic development under *in vivo* and *in vitro* conditions (Shaw et al., 1995; Hidalgo et al., 2003). However, to the best of our knowledge, there have been no studies on the effects of retinol during IVM on the yield of porcine embryos. Moreover, in spite of the beneficial effects of retinol, a suitable dosage for the different retinol metabolites has yet to be determined, since both excessive and inadequate exposure to retinol and its metabolites can be detrimental

for mouse and bovine embryo development (Sporn and Roberts, 1991; Huang et al., 2006). The fifth objective of the present study was to evaluate the effect of both retinol metabolites supplementation at different concentrations in the IVM medium on IVM and IVF parameters and on *in vitro* embryo development quality.

MATERIAL AND METHODS

MATERIAL

Animals

Ovaries were obtained from prepuberal gilts at a local slaughterhouse and transported to the laboratory at 35ºC within 1 h after collection. Only ovaries with follicles 3-6 mm in diameter were selected.

All the cryopreserved semen used in the present thesis belongs to ejaculates of Pietrain boars with proven fertility and normal semen picture used in previous experiment. These boars were chosen based on their in vitro fertilizing potential and subsequently in vitro embryo development to blastocyst stage.

Reagents and culture media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain). The medium used for the collection and washing of COCs was Dulbecco´s Phosphate-Buffered Saline (DPBS) medium composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO4 and 1.46 mM $CaCl_2$-$2H_2O$ supplemented with 4 mg/mL bovine serum albumin (BSA; [fraction V]), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 µg/ml Kanamycin (mDPBS). The oocyte maturation medium was BSA-free North Carolina State University (NCSU)-23 (Peters and Wells 1993) supplemented with 10% (v:v) porcine follicular fluid, 0.1 mg/mL cysteine and 10 ng/mL epidermal growth factor. The basic medium used for fertilization was essentially the same as that used by Abeeydera and Day (1997a). This medium, designated as modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris (crystallized free base), 11 mM glucose, 5 mM sodium pyruvate, and was supplemented with 2mM caffeine and 0.2% BSA (fraction V; A 7888, initial fractionation by cold alcohol

precipitation). The embryo culture medium was a sequential medium based in NCSU-23 supplemented with 0.4% BSA.

METHODS

Recovery and IVM of cumulus-oocyte complexes

Ovaries were obtained from prepuberal gilts at a local slaughterhouse and transported to the laboratory at 35ºC within 1 h after collection in 0.9% NaCl containing 70 µg/mL Kanamycin. After the ovaries were washed three times in NaCl solution, follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle connected to a 10 ml disposable syringe. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times in maturation medium and matured in a 4-well multidish (Nunc, Roskilde, Denmark) containing 150-200 COCs per well in 500 µl of maturation medium supplemented with 10 IU/ml eCG (Folligon, Intervet International B.V., Boxmeer, The Netherlands) and 10 IU/ml hCG (Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain) for 20-22 h and then for another 20-22 h in maturation medium without hormones. Oocyte maturation was carried out under mineral oil at 39ºC in an atmosphere of 5% CO_2 in air.

In vitro fertilization

Fertilization was performed in TBM medium supplemented according to the experimental design. After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium by vortexing for 2 min, 1660 rounds/min. Oocytes were washed twice in maturation medium and three times in pre-equilibrated fertilization medium. Groups of 50 denuded oocytes were then placed in 50 µl drops of fertilization medium in a 35 x 10 mm Petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, USA) under mineral oil and held at 39ºC in an atmosphere of 5% CO_2 in air for about 30 min until addition of spermatozoa.

Sperm Cryopreservation

Ejaculates were cryopreserved using the straw freezing procedure described by Westendorf et al., (1975) as modified by Thurston et al., (1999) and Roca et al., (2003). Briefly, sperm-rich fractions were collected by the gloved-hand method and extended (1:1, v:v) in BTS (Beltsville Thawing Solution; Pursel and Johnson, 19975). The extended

semen was transferred to 50 ml tubes, cooled to 17ºC and sent packaged in insulated containers under suitable conditions to the laboratory. At the laboratory extended sperm-rich fractions were centrifuged (Megafuge 1.0R, Heraus, Germany) for 3 min at 2400 x g (Carvajal et al., 2004). The resulting pellets were re-extended in lactose egg-yolk (LEY) extender (Westendorf et al., 1975) to a concentration of 1.5 x 10^9 cells/mL. After further cooling to 5ºC within 90-120 min, the extended spermatozoa were re-suspended with LEY- Glicerol-OrvusESPaste (LEYGO) extender (Westendorf et al., 1975) to yield a final concentration of 1.5 x 10^9 cells/mL. The cooled spermatozoa were packed into 0.5 mL medium plastic straws (Minitüb, Tiefenbach, Germany), and frozen using a controlled-rate freezer (IceCube 1810, Minitüb, Tiefenbach, Germany) as follows: from 5ºC to -5ºC at a rate of 6ºC/min, from -5ºC to -80ºC a 40ºC/min at 40º C/min, held for 30 s at –80ºC, then cooled at 70ºC/ min to –150ºC and plunged into liquid nitrogen for storage.

Thawing and post-thaw sperm evaluation

Thawing of straws was done in circulating water at 37ºC for 20 s. One pool of semen was made from two straws thawed. The characteristics of frozen-thawed spermatozoa were analyzed 30 min after thawing. Sperm motility was objectively evaluated using a computer assisted sperm analysis (CASA) system (Sperm Class Analyzer ® Microptic, Barcelona, Spain). Sperm motility and acrosome abnormalities were assessed for each replicate in each experiment.

Semen processing, in vitro fertilization and gametes coincubation

For IVF, 100 µl of thawed semen were washed three times by centrifugation at 1900 x g for 3 min in mDPBS. The resulting pellet was resuspended in fertilization medium and after appropriate dilution, 50 µl of this sperm suspension was added to a 50 µl drop of fertilization medium containing the oocytes. The gametes were coincubated at 39ºC in a humidified atmosphere of 5% CO_2 in air during different coincubation times according the experimental design.

***In vitro* culture**

Presumptive zygotes were removed from the fertilization medium and washed three times in pre-equilibrated embryo culture medium. Subsequently, they were transferred to a 4-well multidish (50 zygotes per well), each well containing 500 µl of the

same medium under mineral oil, and cultured at 39ºin 5% CO_2 in air for 12- 15 h to assess fertilization parameters or for 7 days to assess embryo development.

Assessment of maturation, sperm penetration and embryo development

To evaluate maturation and fertilization parameters, oocytes and presumptive zygotes were mounted on slides, fixed in a solution of acetic acid:ethanol (1:3) for 48-72 h at room temperature, stained with 1% lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of 400 x. Maturation rate was assessed at 44 h of IVM. Oocytes with chromosomes at metaphase-II and an extruded polar body were considered mature. Fertilization parameters were evaluated 15 h after insemination. Oocytes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei, with their corresponding sperm tails, and two polar bodies. Degenerated oocytes and oocytes with a broken oolemma or abnormal cytoplasmic appearance were not counted. The fertilization parameters evaluated were:

Penetration: number of oocytes penetrated /total oocytes matured.

Monospermy: number of oocytes containing only one male pronucleus/total penetrated.

Number of spermatozoa/oocyte: number of spermatozoa in penetrated oocytes.

Efficiency of fertilization: number of monospermic oocytes/total inseminated.

In certain experiments, the number of spermatozoa bound to the zona pellucida was counted. After each period of coincubation, according the experimental design, oocytes were washed in DPBS and mounted on slide in 4 µL of a dilution of Glycerol-DPBS (3:1) containing 10 µg/mL Hoechst-33342, followed by examination using fluorescence microscopy.

To examine the ability of embryos to develop to the blastocyst stage *in vitro*, presumptive zygotes were cultured for 7 days. On day 2, cleavage rate (number of embryos cleaved/total cultured) and on day 7, blastocyst formation (number of blastocyst/ total cultured) were evaluated under a stereomicroscope. An embryo which had cleaved to the two-cell stage or beyond was counted as cleaved and an embryo with a clear blastocele was defined as a blastocyst. The total cell number, as an indicator of embryo quality, was evaluated by mounting each blastocyst on a slide in 4 µL of a dilution

of Glycerol-DPBS (3:1) containing 10 µg/mL Hoechst-33342, followed by examination using fluorescence microscopy.

EXPERIMENTAL DESIGN

Objective 1. To evaluate the effect of different sperm:oocyte ratios during IVF, using a short coincubation time of 10 min and different periods of post-coincubation in a medium not appropriate for IVF, on *in vitro* fertilization parameters (Paper 1).

In experiment 1.1, a total of 1624 in vitro matured oocytes, from 4 replicates, were inseminated with frozen–thawed spermatozoa at different sperm:oocyte ratios (2000, 1500, 1000 and 500 sperm:oocyte or 12×10^5, 9×10^5, 6×10^5 and 3×10^5 spermatozoa/mL, respectively) and coincubated for 10 min or 6 h in a 4 × 2 factorial experiment. The oocytes from 10 min of coincubation were washed by mechanical pipetting three times in fresh IVF medium to remove spermatozoa not bound to the zona pellucida, transferred to another droplet and incubated in IVF medium (containing no sperm) for 6 h. The oocytes from the other group remained with the spermatozoa for 6 h. Then all presumptive zygotes were washed and transferred to embryo culture medium for 12 h and fixed and stained to assess fertilization parameters. In experiment 1.2, a total of 1872 in vitro matured oocytes, from three replicates, were inseminated with frozen–thawed spermatozoa at different sperm:oocyte ratios (2000, 1500, 1000 and 500 sperm:oocyte) and coincubated for 10 min. Then the oocytes were washed by mechanical pipetting three times to remove spermatozoa not bound to the zona pellucida, and transferred as follows:

(1) Directly to IVC medium for 18 h (group A);

(2) To fresh IVF medium, containing no sperm, only for 2 h and then washed and transferred to IVC medium for 16 h (group B);

(3) To fresh IVF medium containing no sperm, incubated for 6 h, washed and transferred to IVC medium for 12 h (group C, control).

All presumptive zygotes were fixed and stained to assess fertilization parameters.

Objective 2. To optimize an IVF protocol for individual males, using various additives, caffeine, hialuronic acid (HA) and adenosine to IVF medium and different intervals of gamete coincubation (Paper 2).

In experiment 2.1, two boars (boar A and boar B) were randomly selected to evaluate the effect of caffeine (2 mM), HA (0.5 mg/mL, Bayer, Hyonate vet, Barcelona, Spain) and adenosine (10 μM), alone or in combination, in fertilization medium during sperm–oocyte co-incubation. This experiment was performed using eight different fertilization media:

(1) mTBM + caffeine;
(2) mTBM + caffeine + HA;
(3) mTBM + caffeine + adenosine;
(4) mTBM + caffeine + HA + adenosine;
(5) mTBM;
(6) mTBM + HA;
(7) mTBM + adenosine;
(8) mTBM + HA + adenosine.

A total of 2042 IVM-oocytes from three replicates (1055 from boar A and 987 from boar B) were inseminated, fixed and stained to assess IVF parameters.

In experiment 2.2, in an attempt to increase IVF-efficiency using frozen–thawed spermatozoa from the boars described above, various concentrations of HA (0, 0.5, 1.0 and 1.5 mg/mL) and adenosine (0, 10, 20 and 40 μM) were added to the IVF-medium. This experiment was designed according to the necessities of each male, based on results of experiment 2.1. In this way, spermatozoa of boar A were washed and introduced into the IVF-medium containing HA or adenosine, but in the absence of caffeine. On the contrary, spermatozoa of boar B were introduced into an IVF-medium with either HA or adenosine, and with caffeine. A total of 1961 IVM-oocytes from three replicates (965 from boar A and 996 from boar B) were inseminated, fixed and stained to assess IVF-parameters.

In experiment 2.3, the effect of two periods of sperm–oocyte co-incubation (10 min or 6 h) in each boar was investigated, in an attempt to improve the efficiency of IVF. Based on results of experiments 2.1 and 2.2, spermatozoa were washed and co-incubated with oocytes for 10 min or 6 h in absence (boar A) or in presence (boar B) of caffeine.

Oocytes co-incubated for 10 min were washed three times in fresh fertilization medium using a small pipette to eliminate spermatozoa not attached to the zona pellucida (ZP) and further cultured in IVF-medium (containing no spermatozoa) until the 6 h of insemination was completed. After insemination, presumptive zygotes were washed three times in pre-equilibrated embryo culture medium and cultured for 12–15 h to assess fertilization parameters. A total of 1940 IVM-oocytes from three replicates (945 from boar A and 995 from boar B) were used in this experiment.

Objective 3. To evaluate the effects of ultrashort gamete coincubation time on porcine in vitro fertilization (Paper 3).

In an attempt to evaluate the effect of ultrashort periods of gamete co-incubation during pig in vitro fertilization (experiment 3.1), a total of 2833 in vitro matured oocytes from three replicates were inseminated with thawed spermatozoa and coincubated for 0.25, 1, 2, 3, 7, 10 min, and 6 h. The shortest co-incubation time, 0.25 min (15 s) was confirmed by the time we need to inseminate the oocytes in the microdrop and transfer them immediately to other drops with fresh IVF medium (without spermatozoa). Oocytes coincubated from 0.25 to 10 min were washed three times in fresh fertilization medium using a small bore pipette (approximately 150 µm of diameter) to eliminate spermatozoa not attached to the ZP and further cultured in fertilization medium (containing no spermatozoa) until the 6 h of co-incubation time were completed. After each period of co-incubation, 45–50 oocytes from each group were washed in DPBS and mounted on slide in 4 µl of a dilution of glycerol–DPBS (3:1) containing 10 µg/mL of Hoechst (Hoechst-33342) in order to evaluate the number of spermatozoa bound to the zona pellucida. Spermatozoa were counted by using a fluorescence microscope (magnification ×400). After 6 h, the remaining oocytes from each co-incubation time were cultured in NCSU-23 medium for 12–15 h to assess fertilization parameters.

Objective 4. To study whether the combination of a straw-IVF system and a short coincubation time (10 min) is suitable strategy to decrease polyspermic fertilization and improve in vitro production of porcine blastocysts (Paper 4).

In experiment 4.1, three different IVF system were evaluated: a straw-IVF system with 10 min of coincubation time, a straw-IVF system with 6 h of coincubation and the microdrop-IVF system with 6 h (the traditional IVF system used routinely in most IVF laboratories) in attempt to reduce polyspermic fertilization. Various sperm:oocyte ratios (20000, 30000, 40000, and 50000) were used for the 10 min and 6 h straw-IVF systems.

These ratios were selected from previous experiments (data not shown). A ratio of 1000 spermatozoa per oocyte was used for the microdrop-IVF system. Oocytes from the 10 min straw-IVF system were washed three times in fertilization medium to remove spermatozoa not bound to the zona and transferred to clean microdrops of the same medium (containing no sperm) for 6 h. Six hours after insemination, oocytes from both IVF systems were washed three times in pre-equilibrated embryo culture medium and cultured for 16 h to assess fertilization parameters. A total of 723 *in vitro* matured oocytes, from three replicates, were used in this experiment.

The experiment 4.2 was designed to compare porcine *in vitro* embryo production in two IVF systems, the 6 h microdrop-IVF system (1000 sperm/oocyte) and the 10 min straw-IVF system (30000 sperm/oocyte), based on the results of experiment 4.1. Presumptive zygotes from both systems were cultured in NCSU-23 medium for 168 h to assess embryo development. A total of 471 *in vitro* matured oocytes from three replicates were used in this experiment.

Objective 5. To evaluate the effect of both retinol metabolites, 9-cis retinoic acid and all-*trans* retinol, at different concentrations in the IVM medium on IVM and IVF parameters and *in vitro* embryo development (Paper 5).

In experiment 5.1, various concentrations of RA (0, 5, 50 and 500nM) were added to the maturation medium in an attempt to improve *in vitro* oocyte maturation and *in vitro* embryo development. Maturation rates, IVF parameters and *in vitro* embryo development were evaluated in this experiment. A total of 2161 *in vitro* matured oocytes, from five replicates, were used in this experiment.

In experiment 5.2, the effect of all-*trans* retinol (ROH) at different concentrations (0, 125, 1250 and 12500nM) during *in vitro* maturation on IVM, IVF parameters and *in vitro* embryo development was evaluated. A total of 2141 *in vitro* matured oocytes from five replicates were used in this experiment.

STATISTICAL ANALYSIS

All data were analyzed using SPSS, version 13.0, software package (SPSS Inc., Chicago, III) and differences were considered significant at $P<0.05$. Data from all experiments were assessed by analysis of variance using the MIXED procedure according to a statistical model including the fixed effects and the random effect according each experiment. Maturation, penetration, monospermic rates, efficiency data and blastocyst formation were modelled according to the binomial model of parameters as described by Fisz (1981) before analysis. When analysis of variance showed a significant effect, values were compared using the Bonferroni test. Results are expressed as mean ± SEM.

CONCLUSIONS

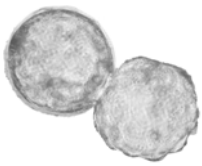
1. The coincubation time may be reduced to 10 min as strategy to increase the efficiency of fertilization depending on sperm:oocyte ratio. Post- incubation of oocytes with the zona-bound sperm in a medium unsuitable for IVF does not improve the efficiency of fertilization. Furthermore, the spermatozoa bound to the zona pellucida require a maximum of 2 h in an appropriate medium to penetrate the oocytes (Paper 1).

2. The presence of caffeine in the IVF-medium has a significant effect on the fertilization efficiency, but with a clear boar influence. Under caffeine or caffeine-free conditions, addition of HA, adenosine or the combination of both to the fertilization medium do not affect the IVF parameters using semen from either boar. The effect of various HA concentrations on IVF parameters depends on the boar used. An evident boar effect on the fertilizing ability of spermatozoa during different coincubation times was found. Thus, the needs of boar spermatozoa for IVF in terms of additives add to IVF medium and coincubation time vary among boars (Paper 2).

3. A co-incubation time as brief as 2 min is long enough to obtain good fertilization rates similar to those achieved from current long-term exposure times (6 h) in porcine IVF. The reduction of co-incubation time from 6 h to 0.25 min does not affect the monospermy or the number of spermatozoa per oocytes (Paper 3).

4. The straw-IVF system in combination with a 10-min coincubation increase monospermic penetration, the efficiency of fertilization and the quality of blastocysts in terms of cell number/embryo as compared with the microdrop-IVF system. In addition, this new strategy allows the use of a wide range of sperm:oocyte ratios (Paper 4).

5. The addition 5nM of *9- cis* retinoic acid in the IVM medium significantly increase blastocyst formation, suggesting that RA may play an important role during IVM (Paper 5).



Bibliografía

Bibliografía 11

Abeydeera LR. 2001. In vitro fertilization and embryo development in pigs. *Reproduction.* 58; Suppl 159–173.

Abeydeera LR. 2002. In vitro production of embryos in swine. *Theriogenology.* 57; 256–273.

Abeydeera LR, Day BN. 1997a. Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen–thawed ejaculated spermatozoa. *Biol. Reprod.* 57; 729–734.

Abeydeera LR, Day BN. 1997b. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. *Theriogenology.* 48; 537–544.

Almiñana C, Gil MA, Cuello C, Roca J, Vazquez JM, Martinez EA. 2005. Adjustments in IVF system for individuals: value of additives and time of sperm-oocyte co-incubation. *Theriogenology.* 64; 1783-1796.

Almiñana C, Gil MA, Cuello C, Parrilla I, Roca J, Vazquez JM, Martinez EA. 2007a. Effects of ultrashort gamete co-incubation on porcine in vitro fertilization. *Anim. Reprod. Sci.* doi:10.1016/j.anireprosci.2007.05.017

Almiñana C, Gil MA, Cuello C, Caballero I, Roca J, Vazquez JM, Martinez EA. 2007b. In vitro fertilization (IVF) in straws and a short gamete coincubation time improves the efficiency of porcine IVF. *Reprod. Domestic. Anim.* doi:10.1111/j.1439-0531.2007.00995.*x*.

Beebe DJ, Wheeler BM, Zeringue H, Walters E, Raty S. 2002. Microfluidic technology for assisted reproduction. *Theriogenology.* 57; 125-135.

Bungum M, Bungum L, Humaidan P. 2006. A prospective study, using sibling oocytes, examining the effect of 30 s versus 90 min gamete co-incubation in IVF. *Hum. Reprod*. 21; 518–523.

Carvajal G, Cuello C, Ruiz M, Vazquez JM, Martinez EA, Roca J. 2004. Effects of centrifugation before freezing on boar sperm cryosurvival. *J Androl.* 25;389-96.

Cheng WTK, Polge C, Moor RM. 1986. In vitro fertilization of pig and sheep oocytes, *Theriogenology*. 25; 146.

Clark SG, Walters EM, Beebe DJ, Wheeler BM. 2002. In vitro fertilization of porcine oocytes in polydimethylsiloxane (PDMS)-glass microchannels. *Biol. Reprod.* Suppl 1; 528

Clark SG, Walters EM, Beebe DJ, Wheeler BM. 2003. A novel integrated in vitro maturation and in vitro fertilization system for swine. *Theriogenology*. 59; 441.

Clark SG, Haubert K, Beebe DJ, Ferguson E, Wheeler BM. 2005. Reduction of polyspermic penetration using biomimetic microfluidic technology during in vitro fertilization. *Lab. Chip.* 5; 1229-1232.

Coy P, Martinez EA, Ruiz S, Vazquez JM, Roca J, Matas C. 1993. In vitro fertilization of pig oocytes alter different coincubation intervals. *Theriogenology.* 39; 1201–1208.

Day BN. 2000. Reproductive biotechnologies: current status in porcine reproduction. *Anim. Reprod. Sci.* 60–61; 161–172.

Duque P, Diez C, Royo L, Lorenzo PL, Carneiro G, Hidalgo, CO, Facal N, Gomez E. 2002. Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. *Hum. Reprod.* 17; 2706-2714.

Fisz M. 1980. Some probability distributions. In: R. De Krieger, Editor, Probability theory and mathematical statistics, John Wiley and Sons, Malabar, FL, USA .129–174.

Fraser LR. 1995. Ionic control of sperm function. *Reprod. Fertil. Dev.* 7; 905–925.

Funahashi H. 2003. Polyspermic penetration in porcine IVM–IVF systems. *Reprod. Fertil. Dev.* 15; 167–177.

Funahashi H, Day BN. 1993. Effects of follicular fluid at fertilization in vitro on sperm penetration in pig oocytes. *J. Reprod. Fertil.* 99; 97–103.

Funahashi H, Day BN. 1997. Advances in in vitro production of pig embryos. *J. Reprod. Fertil.* 52; 271–283.

Funahashi H, Nagai T. 2000. Sperm Selection by a Climbing-over-a-wall method reduces the incidence of polyspermic penetration of porcine oocytes. *J. Reprod. Dev.* 46; 319-324.

Funahashi H, Nagai T. 2001. Regulation of in vitro penetration of frozen–thawed boar spermatozoa by caffeine and adenosine, *Mol. Reprod. Dev.* 58; 424–431.

Funahashi H, Romar R. 2004. Reduction of the incidence of polyspermic penetration into porcine oocytes by pre-treatment of fresh spermatozoa with adenosine and a transient co-incubation of the gametes with caffeine. *Reproduction.* 128; 789–800.

Funahashi H, Cantley TC, Day BN. 1997. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol. Reprod.* 57; 49-53.

Funahashi H, Asano A, Fujiwara T, Nagai T, Niwa K, Fraser LR. 2000. Both fertilization promoting peptide and adenosine stimulate capacitation but inhibit spontaneous acrosome loss in ejaculated boar spermatozoa in vitro. *Mol. Reprod. Dev.* 55; 117–124.

Funahashi H, Fujiwara T, Nagai T. 2000. Modulation of function of boar spermatozoa via adenosine and fertilization-promoting peptide receptors reduce the incidence of polyspermic penetration into porcine oocytes. *Biol. Reprod.* 63; 1157–1163.

Garbers DL, First NL, Sullivan JJ, Lardy HA. 1973. The effects of cyclic nucleotide phosphodiesterase inhibitors on ejaculated porcine spermatozoa metabolism. *Biol. Reprod.* 8; 599–606.

Gil MA, Abeydeera LR, Day BN, Vazquez JM, Roca J, Martinez EA. 2003. Effect of the volume of medium and number of oocytes during in vitro fertilization on embryo development in pigs. *Theriogenology.* 60; 767–776.

Gil MA, Ruiz M, Cuello C, Vazquez JM, Roca J, Martinez EA. 2004a. Influence of sperm:oocyte ratio during in vitro fertilization of in vitro matured cumulus-intact pig oocytes on fertilization parameters and embryo development, *Theriogenology.* 61; 551–560.

Gil MA, Ruiz M, Vazquez JM, Roca J, Day BN, Martinez EA. 2004b. Effect of short period of sperm-oocyte coincubation during in vitro fertilization on embryo development in pigs. *Theriogenology*. 62; 544-52.

Gil MA, Roca J, Cremades T, Hernandez M, Vazquez JM, Rodriguez-Martinez H, Martinez EA. 2005. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates ?. *Theriogenology.* 64; 305–316.

Gil MA, Almiñana C, Cuello C, Parrilla I, Roca J, Vazquez JM, Martinez EA. 2007. Brief coincubation of gametes in porcine in vitro fertilization: Role of sperm:oocyte ratio and post-coincubation medium. *Theriogenology.* 67; 620-626.

Gomez E, Royo LJ, Duque P, Carneiro G, Hidalgo C, Goyache F, Lorenzo PL, Alvarez I, Facal N, Diez C. 2003. 9-cis-retinoic acid during in vitro maturation improves development of the bovine oocyte and increases midkine but not IGF-I expression in cumulus-granulosa cells. *Mol. Reprod. Dev*. 66; 247- 255.

Grupen CG, Nottle MB. 2000. A simple modification of the in vitro fertilization procedure. *Theriogenology*. 53; 422.

Han YM, Abeydeera LR, Kim JH, Moon HB, Cabot RA, Day BN, Prather RS. 1999a. Growth retardation of inner cell mass cells in polyspermic porcine embryos produced in vitro. *Biol. Reprod.* 60; 1110–1113.

Han YM, Wang WH, Abeydeera LR, Petersen AL, Kim JH, Murphy C, Day BN, Prather RS. 1999b. Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos. *Biol. Reprod*. 61; 1340–1346.

Hidalgo CO, Diez C, Duque P, Facal N, Gomez E. 2003. Pregnancies and improved early embryonic development with bovine oocytes matured in vitro with 9-cis-retinoic acid. *Reproduction.* 125; 409- 416.

Huang FJ, Hsuuw YD, Lan KC, Kang HY, Chang SY, Hsu YC, Huang KE. 2006. Adverse effects of retinoic acid on embryo development and the selective expression of retinoic acid receptors in mouse blastocysts. *Hum. Reprod.* 21; 202-209.

Hunter RHF, Dziuk PJ. 1968. Sperm penetration of pig eggs in relation to the timing of ovulation and insemination. *J. Reprod. Fertil.* 15; 199–208.

Hunter RH, Rodriguez-Martinez H. 2004. Capacitation of mammalian spermatozoa in vivo, with a specific focus on events in the Fallopian tubes. *Mol. Reprod. Dev.* 67; 243-250.

Kidson A, Colebrander B, Verheijden JHM, Bevers MM. 2001. Polyspermy in the pig is dependent on both IVF medium and sperm dose during fertilization in vitro, Proceedings of the sixth international conference on pig reproduction University of Missouri, Columbia, MO, p. 74.

Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T. 2002. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. *Biol. Reprod.* 66; 1033-1041.

Li YH, Ma W, Li M, Hou Y, Jiao LH, Wang WH. 2003. Reduced polyspermic penetration in porcine oocytes inseminated in a new in vitro fertilization (IVF) system: straw IVF. *Biol. Reprod.* 69; 580-585.

Lima PF, Oliveira MA, Santos M H, Reichenbach HD, Weppert M, Paula-Lopes FF, Neto CC, Goncalves PB. 2006. Effect of retinoids and growth factor on in vitro bovine embryos produced under chemically defined conditions. Anim. *Reprod. Sci*. 95; 184-92.

Livingston T, Eberhardt D, Edwards JL, Godkin J. 2004. Retinol improves bovine embryonic development in vitro. *Reprod. Biol. Endocrinol.* 21; 2:83.

Marchal R, Pelaez J, Terque M, P. Mermillod. 2002. Effect of sperm survival and CTC staining pattern on in vitro fertilization of porcine oocytes. *Theriogenology.* 57; 1917–1927.

Martinez EA, Vazquez JM, Matas C, Gadea J, Maria IA, Roca J. 1996. Oocyte penetration by fresh or stored diluted boar spermatozoa before and alter in vitro capacitation treatments. *Biol. Reprod.* 55; 134–140.

Martinez-Madrid B, Domínguez E, Alonso C, Diaz C, Garcia P, Sanchez R. 2001. Effect of IVF medium and sperm concentration on fertilization parameters, Proceedings of the sixth international conference on pig reproduction University of Missouri, Columbia, MO, p. 75.

Mattioli M, Bacci ML, Galeati G, Seren E. 1989. Developmental competence of pig oocytes matured and fertilized in vitro. *Theriogenology.* 31; 1201–1207.

McCauley TC, Mazza MR, Didion BA, Mao J, Wu G, Coppola G, Coppola GF, Di Berardino D, Day BN. 2003. Chromosomal abnormalities in day-6, in vitro produced embryos, *Theriogenology.* 60; 1569–1580.

Nagai T. 2000. The improvement of in vitro maturation systems for bovine and porcine oocytes. *Theriogenology* 55; 1291-1301.

Nagai T, Takahashi T, Masuda H, Shioya Y, Kuwayama M, Fukushima M, Iwasaki S, Hanada A. 1988. In vitro fertilization of pig oocytes by frozen boar spermatozoa. *J. Reprod. Fertil.* 84; 585–591.

Niwa K. 1993. Effectiveness of in vitro maturation and in vitro fertilization techniques in pigs. *J. Reprod. Fertil.* 48; 49–59.

Ocampo MB, Ocampo LC, Mori T, Ueda J, Kanagawa H. 1994. Timing of sequential changes in chromosome configurations during the second meiotic division and cytoplasmic events of pig oocytes matured and fertilized in vitro. *Anim. Reprod. Sci.* 34; 281–288. Abstract

Petters RM, Wells KD. 1993. Culture of pig embryos. *J. Reprod. Fertil.* 48; 61–73.

Prathers RS, Day BN. 1998. Practical considerations for the in vitro production of pig embryos. *Theriogenology.* 49; 23–32.

Pursel VG, Johnson LA. 1975. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J. Anim. Sci.* 40; 99-102.

Rath D. 1992. Experiments to improve in vitro fertilization techniques for in vivo matured porcine oocytes. *Theriogenology.* 37; 885–886.

Roca J, Carvajal G, Lucas X, Vazquez JM, Martinez EA. 2003. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen–thawed spermatozoa. *Theriogenology*. 60; 77–87.

Rodriguez–Martinez H, Larson B, Pertoft H, Kjellen L. 1998. GAGs and spermatozoon competence in vivo and in vitro. In: A. Lauria, F. Gandolfi, G. Enne and L. Gianaroli, Editors, Gamete development and function, Serono Symposia, Rome. 239–272.

Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vazquez JM, Martinez E, Roca J, Sanz L, Calvete JJ. 2005. Boar spermatozoa in the oviduct. *Theriogenology*. 63; 514- 535.

Shaw DW, Farin PW, Washburn SP, Britt JH. 1995. Effects of retinol palmitate on ovulation rate and embryo quality in superovulated cattle. *Theriogenology*. 44; 51-58.

Sirard MA, Dubuc A, Bolamba D, Zheng Y, Coenen K. 1993. Follicule-oocyte–sperm interactions in vivo and in vitro in pigs. *J. Reprod. Fertil.* 48; Suppl 3–16.

Sirard MA, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology*. 7; 126-136.

Sporn MB, Roberts A. 1991. Interactions of retinoids and transforming growth factor-beta in regulation of cell differentiation and proliferation. *Mol Endocrinol.* 5; 3-7.

Suzuki K, Ebihara M, Nagai T, Clarke NGE, Harrison RAP. 1994. Importance of bicarbonate/CO2 for fertilization of pig oocyte in vitro, and synergism with caffeine. *Reprod. Fertil. Dev*. 6; 221–227.

Suzuki K, Eriksson B, Shimizu H, Nagai T, Rodríguez-Martinez H. 2000. Effect of hyaluronanon monospermic penetration of porcine oocytes fertilizated in vitro. *Int. J. Androl.* 23; 13–21.

Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodríguez-Martinez H. 2002. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int. J. Androl.* 25; 84–93.

Tienthai P, Kjellén L, Pertoft H, Suzuki K, Rodriguez-Martinez H. 2001. Localisation and quantitation of hyaluronan and sulphated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. *Reprod. Fert. Dev.* 12; 173–182.

Thurston LM, Watson PF, Holt WV. 1999. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. *J. Reprod. Fertil.* 117; 271-80.

Vazquez JM, Martinez EA, Roca J, Coy P, Pastor LM. 1993. Acrosome reaction of boar spermatozoa in vitro fertilization. *Mol. Reprod. Dev.* 36; 4–8.

Wang WH, Niwa K, Okuda K. 1991. In-vitro penetration of pig oocytes matured in cultured by frozen–thawed ejaculated spermatozoa. *J. Reprod. Fertil.* 93; 491–496.

Wang WH, Abeydeera LR, Okuda K, Niwa K. 1994. Penetration of porcine oocytes during maturation in vitro by cryopreserved ejaculated spermatozoa. *Biol. Reprod.* 50; 510–515.

Wang WH, Abeydeera LR, Cantley TC, Day, BN. 1997. Effects of oocyte maturation media on development of pig embryos produced by in vitro fertilization. *J. Reprod. Fertil.* 111; 101-108.

Wang WH, Abeydeera LR, Han YM, Prather RS, Day BN. 1999. Morphologic evaluation and actin filament distribution in porcine embryos produced in vitro and in vivo. *Biol. Reprod*. 60; 1020-1028.

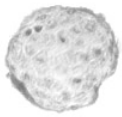
Wang WH, Day BN, Wu GM. 2003. How does polyspermy happen in mammalian oocytes? *Microsc. Res. Technol.* 61; 335-341.

Westendorf P, Richter L, Treu H. 1975. Deep freezing of boar sperma. Laboratory and insemination results using the Hulsenberger paillete method. *Dtsch Tierarztl Wochenschr.* 82; 261-267. German.

Xu X, Seth PC, Harbison DS, Cheung AP, Foxcroft GR. 1996. Semen dilution for assessment of boar ejaculate quality in pig IVM and IVF systems. *Theriogenology.* 46; 1325–1337.

Yokoo M, Tienthai P, Kimura N, Niwa K, Sato E, Rodriguez-Martinez H. 2002. Localisation of the hyaluronan receptor CD44 in porcine cumulus cells during in vivo and in vitro maturation. *Zygote.* 10; 317–326.

Yoshida M. 1987. In vitro fertilization of pig oocytes matured in vivo. *Jpn. J. Vet. Sci.* 49; 711–718.



Anexo Gráfico

Anexo Gráfico 12

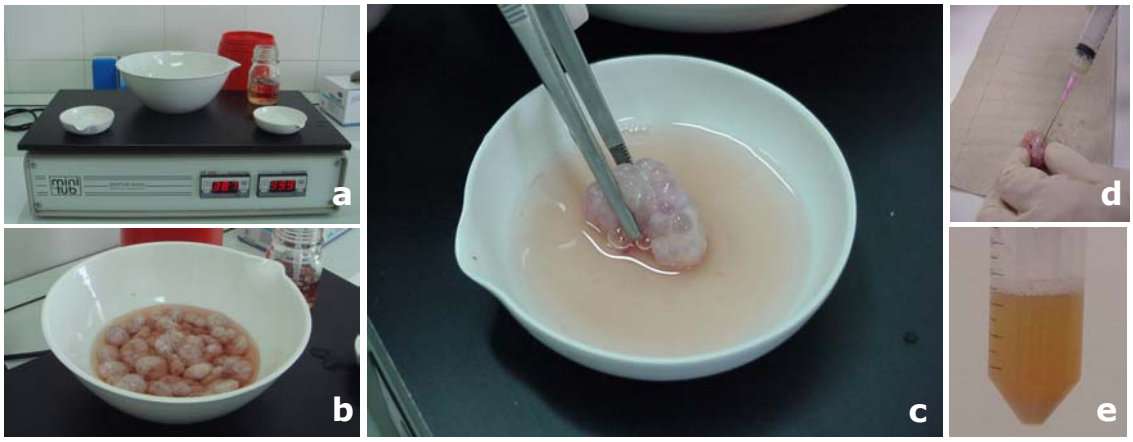


Figura 1. Preparación de los ovocitos para su maduración *in vitro*: a) Placa térmica a 39ºC con medio de lavado. **b)** Ovarios de cerdas prepuberales obtenidos del matadero en medio de lavado. **c)** Detalle de un ovario de cerda prepuberal con folículos de 3-6 mm de diámetro. **d)** Aspiración de los folículos. **e)** Recolección del contenido folicular para su sedimentación.

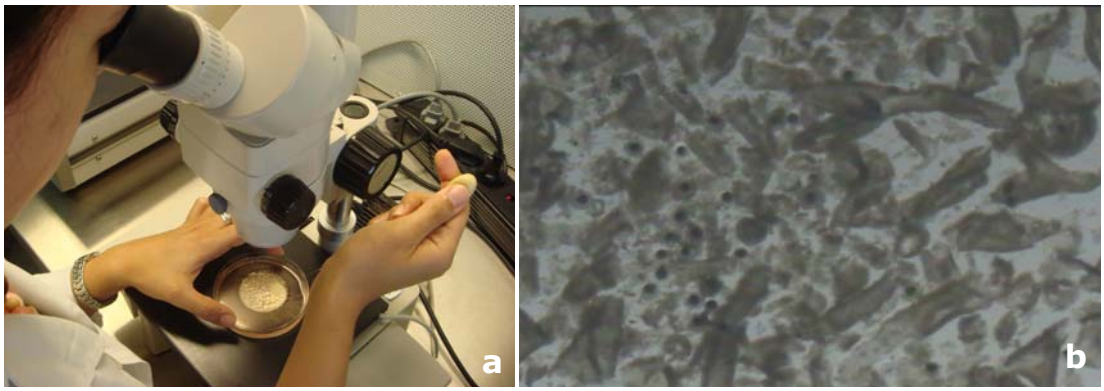


Figura 2. Recolección de los complejos cúmulus-ovocito: a) Visualización y recolección de los COCs mediante un estereomicroscopio. **b)** Contenido folicular visto bajo el estereomicroscopio, obsérvese la presencia de ovocitos inmaduros.

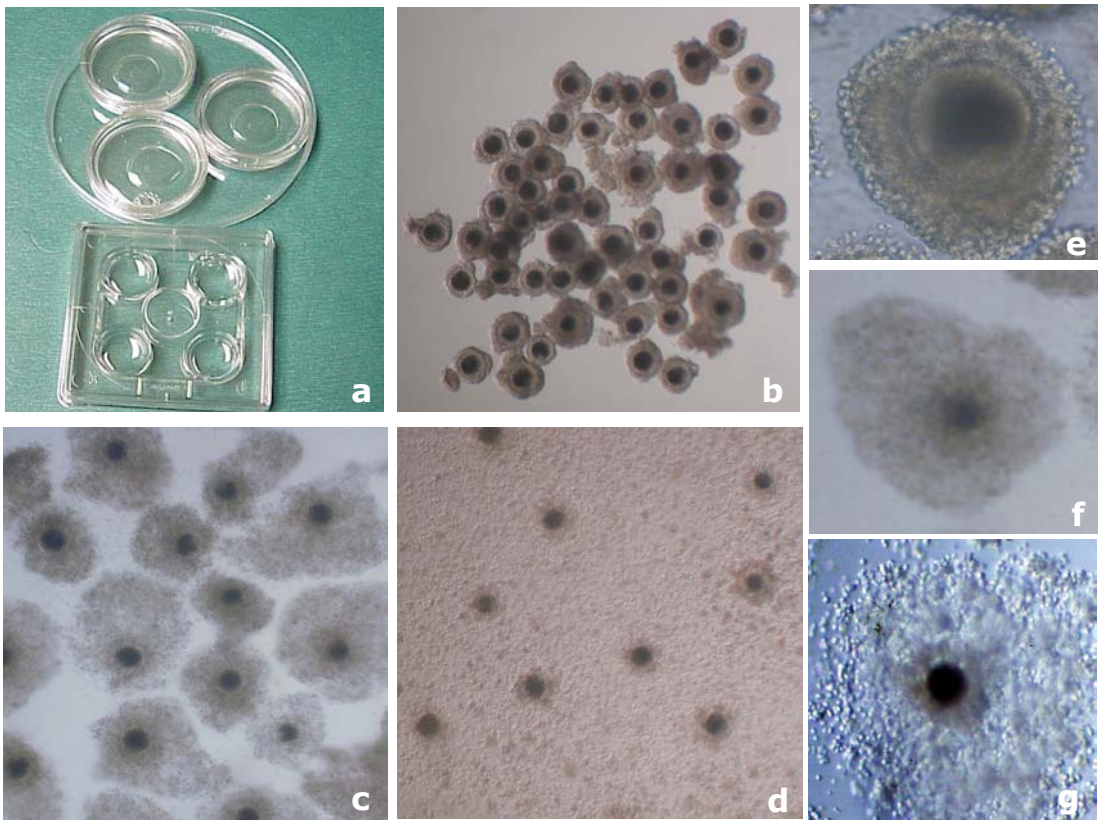


Figura 3. Maduración *in vitro*: a) Placas de Petri de 35 mm para el lavado de los ovocitos y placas Nunc de 4 pocillos para la maduración, ambas con gotas de 500 µl de medio de maduración y cubiertas de aceite mineral. **b)** Ovocitos inmaduros seleccionados para la maduración *in vitro.* **c)** Imagen de los ovocitos en las primeras 24 h del proceso de maduración. **d)** Imagen de los ovocitos maduros a las 44 h del proceso de maduración. **e)** Detalle de un ovocito inmaduro rodeado de varias capas de células del cúmulus. **f)** Detalle de un ovocito a las 24 h de la maduración, obsérvese la expansión de las células del cúmulus. **g)** Detalle de un ovocito maduro.

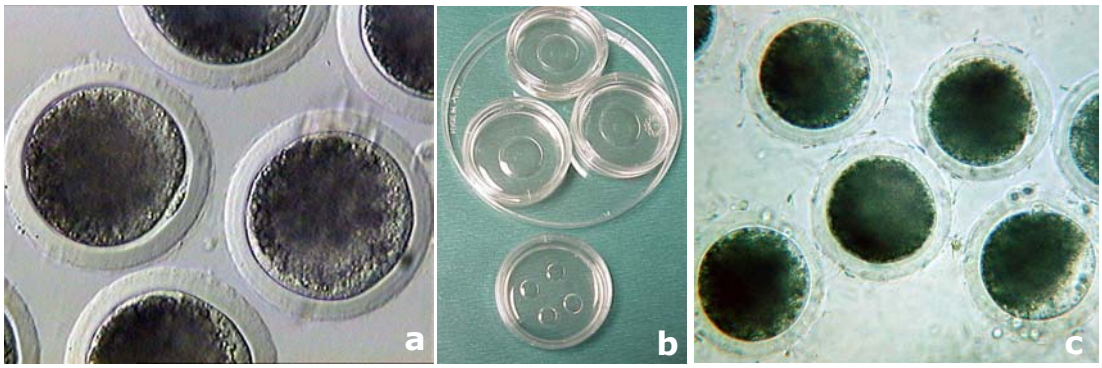


Figura 4. Fecundación *in vitro*: a) Ovocitos denudados preparados para la fecundación. **b)** Placas de Petri de 35 mm con gotas de 500 µl y placas con 4 gotas de 50 µl cada una con de medio de fecundación y cubiertas de aceite mineral. **c)** Detalle de los ovocitos fecundados con espermatozoides pegados a la zona pelúcida de los ovocitos.

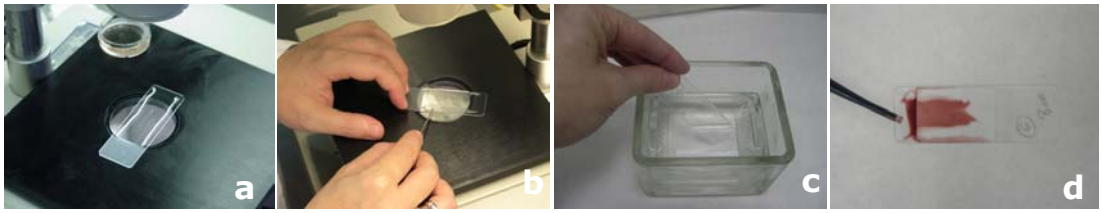


Figura 5. Fijación y tinción de los ovocitos. a) y b) Fijación de los ovocitos entre porta y cubre pegados con finas líneas de vaselina. **c)** Inmersión del porta en acético: etanol (1:3) durante el proceso de fijación. **d)** Tinción de los ovocitos mediante Lacmoid para posterior visualización y análisis de los parámetros de fecundación.

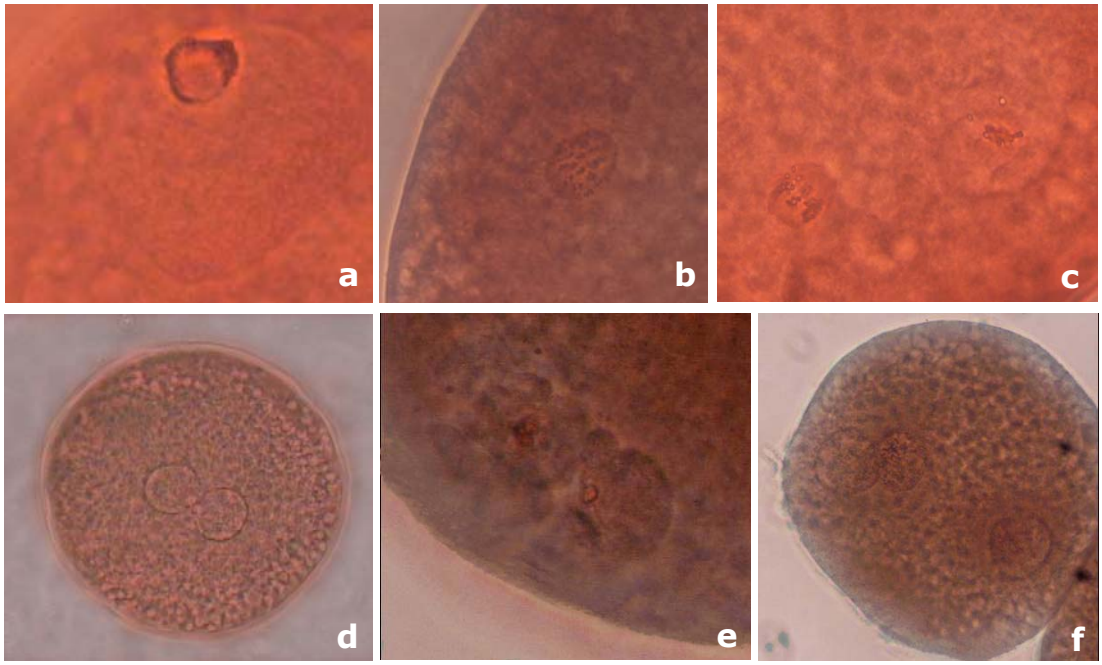


Figura 6. Visualización de los ovocitos teñidos con Lacmoid al microscopio para su evaluación. a) Ovocito inmaduro en estadio de vesícula germinal. **b**) Ovocito inmaduro en estadio de metafase I, obsérvese la placa metafásica. **c)** Ovocito maduro en estadio de metafase II, obsérvese la placa metafísica y el cuerpo polar. **d)** Ovocito monospérmico, con dos pronúcleos en el centro. **e)** Detalle de dos cuerpos polares. **f)** Ovocito polispérmico, obsérvense los tres pronúcleos.

Figura 7. Visualización de los embriones al estereomicroscópio. a) Embriones a las 48 h del cultivo *in vitro* en estadio de 2-4 células. **b)** Imagen del estereomicroscopio en la cabina de flujo. **c)** Embriones a los 7 días del cultivo *in vitro* en estadio de blastocisto.

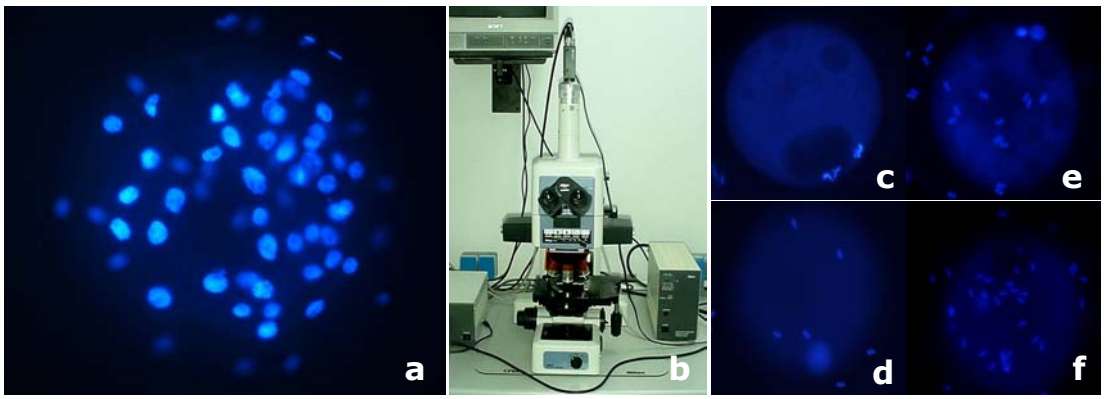


Figura 8. Visualización de los embriones y ovocitos teñidos con Hoechst-33342 al microscopio de fluorescencia. a) Embrión en estadio de blastocisto, obsérvese las células teñidas de Hoechst. **b)** Imagen del microscopio de fluorescencia. **c), d), e) y f)** Ovocitos teñidos tras diferentes tiempos de coincubación con los espermatozoides, obsérvese como varía el número de espermatozoides según el tiempo de coincubación: 0'25 min, sin espermatozoides con la placa metafásica teñida y el cuerpo polar (**a**); 1 min, con 6 espermatozoides unidos a la zona pelúcida (**d**); 7 min, con 17 espermatozoides unidos a la zona pelúcida (**e**); 6 h con 29 espermatozoides unidos a la zona pelúcida (**f**).

Apéndice

UNIVERSIDAD DE MURCIA

Departamento de Medicina y Cirugía Animal

FACULTAD DE VETERINARIA

Campus Universitario de Espinardo

30071 MURCIA

Murcia, 18 de Septiembre de 2007,

A la Comisión General de Doctorado,

Los abajo firmantes, DECLARAN:

Que el trabajo realizado en la tesis: "**Optimización del sistema de fecundación in vitro en la especie porcina: Condiciones de maduración y de cocultivo de los gametos"** es original y ha sido realizado fundamentalmente por el autor de la misma, Carmen Almiñana Brines. Los co-autores, certifican que los artículos contenidos en la presente tesis, no serán presentados en ninguna otra tesis.

1. Emilio A. Martínez García
2. Juan M. Vázquez Rojas
3. Jordi Roca Aleu
4. María A. Gil Corbalán
5. Cristina Cuello Medina
6. Inmaculada Parrilla Riera
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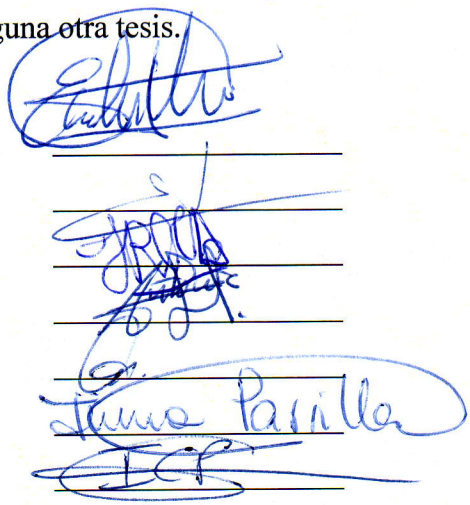

SVERIGES LANTBRUKSUNIVERSITET
Fakulteten för Veterinärmedicin och Husdjursvetenskap
Avdelningen för komparativ reproduktion, obstetrik och juverhälsa

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES
Faculty of Veterinary Medicine and Animal Science
Division of Comparative Reproduction, Obstetrics and Udder Health

Professor Heriberto Rodríguez-Martínez

Datum/Date

2007-09-18

A quién le pueda interesar,

Dr. Heriberto Rodríguez-Martínez, catedrático y responsable de la Unidad de Ginecología y Reproducción, de la Facultad de Veterinaria de la SLU (Uppsala, Suecia), DECLARA:

Que el trabajo realizado por la doctoranda Carmen Almiñana Brines durante su estancia en nuestro departamento y bajo mi supervisión directa, ha sido realizado mayoritariamente por ella. Asímismo certifico que ninguno de los artículos que aparecen en su tesis serán incorporados en ninguna otra tesis doctoral.

Uppsala, 18 de Septiembre de 2007

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>It is a pleasure to accept your manuscript entitled "In vitro
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Title	In vitro maturation of porcine oocytes with retinoids improv...
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Abstract	The effect of retinoid metabolite administration during in vitro maturation (IVM) on oocyte maturation, in vitro fertilization (IVF) parameters and embryo development was examined. Varying concentrations of 9-cis retinoic acid (RA; 0, 5, 50 and 500nM; Experiment 1) and all-trans retinol (ROH; 0, 125, 1250 and 12500nM; Experiment 2) were included. Cumulus-oocyte complexes were matured in vitro and inseminated with frozen-thawed spermatozoa. Presumptive zygotes were cultured for 16h to assess IVF parameters or for 7 days to assess embryo development and quality. In experiment 1, oocyte maturation rates were significantly decreased ($p<0.001$), with values below 5%, when the highest concentration of RA (500nM) was used, whereas 5 and 50nM RA had no effect compared with the control. The 5nM RA treatment improved blastocyst development rate ($p<0.001$). In experiment 2, oocyte maturation rate did not differ between 125nM, 1250nM ROH and the control, but 12500nM ROH was deleterious. Penetration rate was lower for 1250 than for 125 nM ROH or the control, but blastocyst formation rate did not differ among the three maturation treatments. In conclusion, 5nM RA in the IVM medium significantly increased blastocyst formation rate, suggesting that RA may play an important role during IVM.
Category	Reproductive technologies and cloning
Invited Submission	no

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Ignacio Caballero	4
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Juan Maria Vazquez	6
Enrique Gomez	7
*** Martinez Emilio A**	8

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Author Suggested Reviewer(s)

none

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