Effect of several approaches to visualize the metaphase II plate and the first polar body on the developmental ability of *in vitro*-mature porcine oocytes.

Efecto de diversas técnicas para visualizar la placa metafásica y el corpúsculo polar sobre la capacidad de desarrollo de ovocitos porcinos madurados *in vitro*.


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

AUTORIZAN

A la Licenciada en Veterinaria Dña. Carolina Maside Mielgo a presentar la Tesis Doctoral titulada “Effect of several approaches to visualize the metaphase II plate and the first polar body on the developmental ability of in vitro-matured porcine oocytes”, (“Efecto de diversas técnicas para visualizar la placa metáfásica y el corpúsculo polar sobre la capacidad de desarrollo de ovocitos porcinos madurados in vitro”), 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 Doctora en Veterinaria. La Tesis es un compendio de cuatro artículos publicados en revistas internacionales incluidas en el primer tercio del JCR, todas ellas de gran difusión en el área de la Reproducción Animal. Los cuatro artículos presentan un cuerpo y unos objetivos comunes dentro del campo de la producción in vitro de embriones porcinos, lo cual justifica la presentación en el formato de compendio de publicaciones. Este formato permite plasmar con facilidad el recorrido realizado por el doctorando y se ajusta al modelo de tesis presentadas actualmente dentro de nuestro área en el ámbito internacional.

Para que conste a los efectos oportunos, emitemos este informe en Murcia a 26 de julio de 2012.

Fdo.: Emilio A. Martínez García

Fdo.: María Antonia Gil Corbalán
D. Fernando Tecles Vicente, Profesor Titular de Universidad del Área de Conocimiento de Medicina y Cirugía Animal y Presidente Comisión Académica programa doctorado * "Medicina y Reproducción Animal", INFORMA:

Que una vez evaluado, de conformidad con el procedimiento establecido en el artículo 21 del Reglamento de doctorado de la Universidad de Murcia, el expediente completo de la tesis doctoral titulada “Efecto de diversas técnicas para visualizar la placa metafásica y el corpúsculo polar sobre la capacidad de desarrollo de ovocitos porcinos madurados in vitro”, realizada por Dª Carolina Maside Mielgo, bajo la inmediata dirección y supervisión de D. Emilio A. Martínez García y Dª María Antonia Gil Corbalán, esta Comisión Académica, en sesión celebrada en fecha 11 de octubre de 2012, ha dado su autorización para su presentación ante la Comisión General de Doctorado.

Murcia, a 11 de octubre de 2012

Mod: T-40
Dª CAROLINA MASIDE MIELGO  
C/ Pío Baroja, 5, 2º C  
30011 Murcia

Vista la solicitud presentada el día 14 de septiembre de 2012, por Dª  
CAROLINA MASIDE MIELGO, con DNI número 48.481.167-L, 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 Presidente de la Comisión Académica del Doctorado en  
Medicina y Reproducción Animal, responsable de la autorización de la tesis  
doctoral en fase de elaboración, de esta Universidad, y  
- el visto bueno de la Comisión de Ramas de Conocimiento de Ciencias de la  
Salud,

resolvió, en su sesión de 4 de octubre de 2012, ACCEDER a lo solicitado por la  
interesada 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 Dª CAROLINA MASIDE MIELGO, 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, 4 de octubre de 2012   
Vicerrectora de Estudios y  
Presidenta de la Comisión General de Doctorado

[Signature]

Concepción Palacios Bernal
To whom it may concern

I have reviewed the work performed by Ms Carolina Maside Mielgo for her PhD thesis entitled “Effect of several approaches to visualize the metaphase-II plate and the first polar body on the developmental ability of in vitro-matured porcine oocytes”. The thesis is of a coat type built upon four articles published in international ranked journals with peer-review system. The coat contains an introduction, set of general objectives, an extended summary including methodological considerations, experimental design and results followed by general conclusions, abbreviations and references.

In her thesis the Candidate has studied a topic which is timely and up to date in the field of veterinary reproduction, particularly in reproductive biotechnology and of interest for other species than the pig, which was her model animal. Diminishing the handling and exposure to unnecessary chemicals to identify genomic material in oocytes is a pre-requisite for the success of SCNT, among other technologies. The difficulties in identifying and imaging the genomic plate in oocytes are a major problem and ways of waiving this difficulty was the major goal of the thesis. Elegantly, the thesis evolved into the use of non-invasive, chemical-free imaging using PLM to detect microtubule polymerized proteins thus aiding for the accurate localization of the metaphase plate and of 1st polar body. Papers, having the candidate as 1st author in two of them, document this path of reasoning and action. The design of the different experiments was always clear and allowed to answer to the initial hypotheses. Data were correctly analysed with and were clearly presented and well-illustrated. The meanings and the implications of the results were clearly put in the context of the current literature and were correctly discussed paper by paper, reaching relevant conclusions. The latter would probably lead into new areas of research which the candidate appears to be well-prepared for.

On the basis of the considerations above, I fully support Ms Maside Mielgo´s candidacy to the European Doctorate.

Prof Dr Heriberto Rodríguez-Martínez
Dept of Clinical & Experimental Medicine
Linköping University, Sweden
Whom it May Concern

Comprehensive evaluation of Carolina Maside Mielgo PhD thesis “Effect of approaches to visualize the metaphase II plate and the first polar body on the developmental ability of in vitro-mature porcine oocytes” as a whole

The main purpose of the thesis was the evaluation of different procedures of MII oocytes visualization (PB1 and MII plate) and their usefulness and possible detrimental effects on the ability of the oocyte to be fertilized and later embryonic development to blastocyst stage in vitro. Although some of the methods considered in the thesis were investigated before by other authors, so far these were not examined carefully in case of porcine oocytes. The necessity of undertaking such studies on pigs is given by the desire to increase efficiency of IVF and/or nuclear transfer procedures what determines using pig as animal model for medical studies.

The obtained results bring some valuable solutions, with particular importance of using polarized light microscopy for improving porcine oocyte enucleation technique, which is known as a trouble generating step during SCNT. Even if its use for non-invasive selection of highly competent individual oocytes seems to be restricted, undertaking this issue was interesting in the context of recent trends in research, focused on searching for reliable oocyte quality markers. Analyses which were carried out revealed also important information about detrimental effects of SYBR-14 staining and combined Hoechst342/UV treatment on mitochondria distribution and other porcine oocyte quality parameters.

The thesis is logically structured with coherent literature background properly introducing the reader into the investigated problems. The relevant methods were chosen and thoroughly performed and described. The obtained results were critically evaluated/interpreted and discussed. Finally, the conclusions which were drawn, closely correspond to the raised objectives. The text is written in proper style and language.

Since this doctoral thesis presents significant benefaction for oocyte manipulation process in porcine species and it can have influence on future pig reproduction biotechnology including IVF and nuclear transfer, I fully recommend this thesis for a doctoral degree and suitable examination.

Yours sincerely,

[Signature]

October, 2nd, 2012

Adam J. Ziecik Ph D, D Sci.
Professor, member of Polish Academy of Sciences
Evaluation Report

of the Thesis by:

Dr. Carolina Maside Mielgo

entitled:

Effect of several approaches to visualize the metaphase II plate and the first polar body on the developmental ability of in vitro mature porcine oocytes

Current knowledge

Somatic cell nuclear transfer (SCNT) is a useful tool for biomedical research. In pigs, the efficiency of SCNT is still very low, although the number of cloned pigs increases each year. The success of the SCNT procedure depends upon numerous factors. One key step is the enucleation of the recipient oocyte. Proper enucleation is essential in that it influences the developmental ability of the SCNT embryos. Porcine oocytes are especially difficult to enucleate because their cytoplasm contains many lipid droplets.

Objectives of the thesis

The main objective of this thesis was to assess the effect of several approaches used to visualize the MII plate and PB1 on in vitro fertilization and subsequent embryo development of porcine oocytes matured in vitro in order to establish an efficient enucleation method that does not compromise the developmental competence of the oocytes.

The results of the experiments have already been published in international journals. The papers are entitled:

- Effects of Hoechst 33342 staining and ultraviolet irradiation on the developmental competence of in vitro-matured porcine oocytes (Experiment 1).
- Effects of Hoechst 33342 staining and ultraviolet irradiation on mitochondrial distribution and DNA copy number in porcine oocytes and preimplantation embryos (Experiment 2).
• Exposure of in vitro-matured porcine oocytes to SYBR-14 and fluorescence impairs their developmental capacity (Experiment 3).

• Use of polarized light microscopy in porcine reproductive technologies (Experiment 4).

Results

The results of the four experiments indicate that:

- The exposure of porcine oocytes to the combination of H342 staining with UV irradiation has a clear deleterious effect on the developmental competence of the oocytes, with the effects being more intense with increased exposure to UV irradiation.

- The exposure of the oocytes to H342 staining and UV irradiation is associated with abnormal mitochondrial distribution pattern and with reduced mtDNA copy number in the resulting cleaved embryos.

- The combination of SYBR-14 staining and fluorescence for periods as short as 5 seconds exerts a clear deleterious effect on the viability and reduces their subsequent developmental competence.

- Polarized Light Microscopy (PLM) is an efficient system to detect microtubule-polymerized protein in in vitro-matured porcine oocytes with no detrimental effects on porcine oocyte developmental competence. PLM appears as an efficient method to enucleate porcine oocytes.

Overall judgement

The thesis is very interesting, original, well prepared and provides very useful information in the field of porcine reproductive technologies. An appropriate experimental design and appropriate methods have been used. The interpretation of the results in the discussion is very good and matches the standards of international research. The results of the thesis have been published in international journals of good standard.

For all these reasons I think that the thesis has been excellently performed and fulfils the requirements of an European PhD Thesis.

Ozzano dell'Emilia 09.10.2012

Prof.ssa Giovanna Galeati
Dear Sir or Madam

Carolina Maside

I am pleased to host Carolina Maside in my laboratory from 1 October to 31 December 2011. During this period she will take part in bioinformatics analysis of microarray experiments. This aims to establish the response of the maternal tract to various sex chromosome-bearing spermatozoa by identifying the differentially altered genes.

In the initial month of her stay she will master bioinformatics analysis of microarrays. In the second and third months she will be involved in designing and constructing primers to validate altered genes by using real time PCR.

Please contact me if you require further information.

Yours faithfully

Alireza Fazeli
Departamento de Medicina y Cirugía animal, Facultad de Veterinaria
Campus de Excelencia Internacional Regional “Campus Mare Nostrum”
Universidad de Murcia
Este trabajo ha sido financiado por
la Fundación Séneca
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“Soy de los que piensan que la ciencia tiene una gran belleza. Un científico en su laboratorio no es sólo un técnico: es también un niño colocado ante fenómenos naturales que le impresionan como un cuento de hadas”.

*Marie Curie, física y química polaca (1867 – 1934).*
A mi madre y mi padre,
Lola y Manuel.
A mis hermanas y mis sobrinas,
Pili, Evita, Carolina, Lucía, Evita y Víqui.
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Este trabajo no se habría convertido en una realidad sin la ayuda inestimable, apoyo y comprensión de multitud de personas que, en mayor o menor medida, directa o indirectamente, han colaborado incansablemente en la realización de esta tesis, leyendo, opinando, corrigiendo, teniendo mucha paciencia, acompañándome en los momentos de crisis y en los momentos de felicidad, sin exasperación por su parte, sobre todo al inicio del trabajo, cuando los conocimientos eran prácticamente inexistentes.

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A la Dr. Xiomara Lucas, mi compañera y sobre todo mi amiga, por los mejores consejos que nunca me han dado, y porque no es necesario que hablemos para saber como estamos. Porque además de ser una grandísima profesional, eres una de las mejores personas que he conocido, y si por algo ha merecido todo esto, es porque formas parte de mi vida. ¡Ay Mari! Como te quiero!

A mis “compis” de despacho, las Dra. Cristina Cuello y Dra. Inmaculada Parrilla, que son un ejemplo a seguir por su capacidad de lucha, trabajo y tenacidad. A vuestro lado durante todos estos años, no sólo he evolucionado profesionalmente sino también personalmente. Gracias a vuestro apoyo, vuestros grandes consejos, por compartir contigo vuestros conocimientos, vuestra pasión y entrega total a la investigación, por las mil y una charla que tanto me han ayudado y animado a seguir adelante, nuestros abrazos matutinos, los cafés mañaneros, las confesiones, y los innumerables momentos que hemos compartido.

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A Eugenia, Luz María y Maribel, mis ángeles de la guarda, gracias por existir y estar en mi vida, por quererme tal y como soy aunque os lo haya puesto en ocasiones un tanto difícil, por demostrarme cada día que la palabra amistad la puedo escribir con letras mayúsculas. Sin vosotras mi sueño no lo habría cumplido. Gracias a vosotras en este camino he podido sentirme de mil maneras, pero nunca sola; Siempre nos quedará Wisconsin!

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Introduction.................................................................3
Objectives.................................................................9
Extended summary......................................................13
Conclusions..............................................................35
Resumen General.........................................................39
Abbreviations............................................................71
References...............................................................75
Articles.................................................................85
Graphic Appendix.......................................................135
Appendix...............................................................151
INTRODUCTION
INTRODUCTION

In the last years, somatic cell nuclear transfer (SCNT) technology has been widely used to clone several mammalian species, including cattle, mice, goats, pigs, cats, rabbits, horses, rats, dogs and ferrets (reviewed in Meissner and Jaenisch, 2006; Campbell et al., 2007). In particular, pig cloning by SCNT has become a useful tool for the elaboration of genetic models for human diseases and the use in xenotransplantation (Dai et al., 2002; Lai et al., 2002; Fujimura et al., 2008; Rogers et al., 2008; Yazaki et al., 2009). Although the number of cloned piglets derived from somatic cells has continuously increased since it was first published in 2000 (Betthauser et al., 2000; Onishi et al., 2000; Polaejaeva et al., 2000), this method still has a low overall efficiency rate (reviewed in Vajta et al., 2007).

The SCNT procedure involves several steps and factors, each of which may have an influence on the efficiency of the technique, such as the state and type of the donor cell, source and quality of oocytes, the preparation and manipulation of the recipient cytoplasm, timing and methods of manipulation and activation and finally, embryo culture conditions. One of the hardest steps of porcine SCNT is the enucleation of the recipient oocyte because their cytoplasm contains many lipid droplets (Genicot et al., 2005), becoming the chromosomes invisible to standard microscopy.

Although several approaches have been described for enucleation of porcine oocytes, a procedure implicating the use of bisbenzimide Hoechst 33342 (H342) staining and ultraviolet (UV) irradiation is frequently used to aid the localization and visualization of the metaphase II (MII) plate and the first polar body (PB1) before enucleation (Estrada et al., 2008; Lee et al., 2008; Hickley et al., 2011) or to confirm the enucleation of recipient oocytes after manipulation (Kragh et al., 2005; Das et al., 2010; Koo et al., 2010; Biswas et al., 2011). The H342 is a UV-excitable fluorochrome (excitation 350 nm) and live-cell permeant stain. This dye has widely been used to label oocyte chromatin because H342 strongly binds to the minor groove of DNA (Robison et al., 1998).

Although live piglets have been born from oocytes enucleated in the presence of H342 and UV light, it is widely assumed that the exposure of the recipient oocytes to H342
and UV irradiation might have detrimental effects on their DNA and cytoplasmic organelles (Li et al., 2004). However, the exact toxicity of H342 and UV irradiation on the oocyte is not yet fully known because only a limited number of studies have been conducted in a few species, including cattle, goat, rabbits and mice (Tsunoda et al., 1988; Yang et al., 1990; Westhusin et al., 1992; Smith et al., 1993; Dominko et al., 2000; Velilla et al., 2002; Versieren et al., 2010). These studies showed variable results depending mainly on the species and the length of irradiation. Despite the extensive use of H342 and UV light to enucleate porcine oocytes no studies to date have investigated its effects on porcine oocytes developmental competence.

If H342 staining and UV irradiation can be detrimental for oocyte developmental competence, it would be important to find an alternative method to visualize the maternal chromosome of mature oocytes which allows to remove very little amount of cytoplasm surrounding the spindle and preserve the oocyte volume without compromising the enucleation efficiency and development ability. In that sense, Dominko et al. (2000) reported that SYBR-14, a membrane-permeant nucleic acid stain (485-nm excitation), could be employed as an alternative fluorochrome to H342 because SYBR-14 did not compromise the developmental potential of the bovine oocytes post-activation. The use of a longer-wavelength fluorochrome should allow to visualize the maternal chromosome with lower energy transfer and, therefore, resulting in less harm to the oocytes. However, to our knowledge, no studies to date have investigated such effects in other species, including pigs.

Another possible alternative to visualize maternal chromosomes of porcine oocytes could be the use of an inverted microscope equipped with a polarized light microscopy (PLM) also called as the spindle view system. In the MI oocytes, the meiotic spindle microtubules are macro-molecular; hence, they can refract polarized light. Compared with methods of spindle evaluation that require oocyte fixation, PLM offers the unique advantage of being totally non-invasive, preserving oocyte viability and allowing repeated observations of the sample. PLM principles and equipment have been extensively described elsewhere (Oldenbourg et al., 1996; Wang et al., 2001; Keefe et al., 2005; Shen et al., 2008; Caamaño et al., 2010). The presence of the meiotic spindle appears to be associated with an increased fertilization rate and embryo quality in mouse and human (Wang et al., 2001, 2002; Coticchio et al., 2004). Interestingly, more oocytes with visible spindles fertilized and
progressed to blastocysts compared to oocytes without visible spindles (Raju et al., 2007). In addition, spindle retardance has been proposed as a marker of oocyte quality in human oocytes and it could be used to select oocytes with an improved embryonic developmental competence (Liu et al., 2000; De Santis et al., 2005; Kilani et al., 2009). The structures and conformation of the meiotic spindles are important to predict the fate of the oocytes. If PLM is able to detect spindle abnormalities, it might become a valuable tool to select oocytes with normal spindle morphology and potentially higher developmental competence. Research in PLM has been carried out mainly in mice and human oocytes. However, information on the practical use of PLM in oocytes from farm animals, including pigs, is limited and its potential applications in animal reproduction need further assessment.

Based on all stated above, the present work was designed with the purpose of studying the effect of several approaches to visualize the metaphase II plate and the first polar body on the developmental ability of in vitro-matured porcine oocytes.
OBJECTIVES
OBJECTIVES

The main objective of this thesis was to assess the effect of several approaches used to visualize the metaphase II plate and the first polar body on \textit{in vitro}-fertilization and subsequent embryo development of porcine oocytes matured \textit{in vitro}. With this purpose, the specific objectives contained in this thesis were:

1. To evaluate the effects of exposure to Hoechst 33342 staining and ultraviolet irradiation on fertilisability and subsequent developmental capacity of porcine oocytes matured \textit{in vitro}. (Article 1).

2. To evaluate the effects of Hoechst 33342 staining and ultraviolet irradiation on mitochondrial distribution and mitochondrial DNA copy number in developing porcine oocytes and preimplantation embryos. (Article 2).

3. To investigate the effectiveness of a longer-wavelength fluorochrome (SYBR-14) for visualizing maternal chromosomes in \textit{in vitro}-matured porcine oocytes and the effects of this dye in combination with fluorescence excitation on the subsequent \textit{in vitro} fertilization and embryo development of the oocytes. (Article 3).

4. To assess the efficiency of Polarized Light Microscopy to detect microtubule-polymerized protein and to assist in the removal of the meiotic spindle of \textit{in vitro}-matured porcine oocytes and to examine the effects of Polarized Light Microscopy on the oocyte developmental competence. (Article 4).
EXTENDED SUMMARY

MATERIAL AND METHODS

In vitro maturation and fertilization of oocytes and in vitro culture of early embryos

Collection and culture of cumulus-oocytes complexes

Ovaries were obtained from prepubertal gilts in 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. The cumulus-oocytes complexes (COCs) were aspirated from medium-sized follicles (3 to 6 mm in diameter) using an 18-gauge needle connected to a 10-mL disposable syringe. Dulbecco’s phosphate-buffered saline (DPBS) medium was used for the collection of COCs and for washing; this medium is composed of 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.5 mM CaCl₂·2H₂O and was supplemented with 4 mg/mL bovine serum albumin fraction V (BSA; Sigma-Aldrich Quimica SA, Madrid, Spain), 0.3 mM sodium pyruvate, 5 mM D-glucose and 70 µg/mL kanamycin (mDPBS). Oocytes with a compact cumulus mass and evenly granulated cytoplasm were washed three times in maturation medium. The oocyte maturation medium was BSA-free North Carolina State University medium (NCSU-23; Petter and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid (FF), 0.8 mM cysteine, 10 ng/mL epidermal growth factor and 5 nM 9-cis retinoic acid. The porcine FF was collected from 3- to 6-mm diameter follicles from ovaries obtained as described above, and centrifuged at 1500 x g for 30 min at 15°C. The supernatant was filtered through 0.8-, 0.45-, and 0.22-µm filters, and aliquots were stored at -20°C until use. The COCs were matured in 4-well multidish (Nunc, Roskilde, Denmark) containing 70 to 80 COCs in 500 µL of maturation medium supplemented with 10 IU/mL of equine chorionic gonadotrophin (Folligon, Intervet International B.V., Boxxmeer, the Netherlands) and 10 IU/mL of human chorionic gonadotrophin (VeterinCorion, DivasaFarmavic, S.A., Barcelona, Spain). The oocytes remained in these wells for 20 to 22 h and were then incubated for another 20 to 22 h in maturation medium without hormones. Oocyte maturation was carried out under mineral oil at 39°C in a humidified atmosphere of 5% CO₂ in air. After
maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium by vortexing for 2 min at 1660 rounds/min and were washed twice in maturation medium.

In vitro fertilization and in vitro culture

After maturation, oocytes were washed three times in pre-equilibrated modified Tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl$_2$·2H$_2$O, 20 mM Tris (crystallized free base), 11 mM glucose and 5 mM sodium pyruvate and supplemented with 0.5 mM caffeine and 0.2% BSA (Abeydeera and Day, 1997). In vitro fertilization was performed as described by Gil et al. (2003). Briefly, the oocytes were transferred in groups of 30 denuded oocytes were to 50-µL drops of the same medium in a 35 x 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 approximately 30 min until the addition of spermatozoa. Semen from a mature Pietrain boar was processed and cryopreserved in 0.5-mL straws as described by Carvajal et al. (2004). For each replicate, one pool of semen was made from two straws thawed in a circulating water bath at 37°C for 20 s. Next, 100 µL of thawed semen was washed three times by centrifugation at 1900 x g for 3 min in mDPBS. The resulting pellet was resuspended in mTBM, and 50 µL of this sperm suspension containing 30000 spermatozoa 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 39°C in a humidified atmosphere of 5% CO$_2$ in air for 6 h. The presumptive zygotes were removed from the fertilization medium, washed by mechanical pipetting three times in pre-equilibrated embryo culture medium (NCSU-23 with 0.4% BSA) to remove spermatozoa not bound to the zona and transferred into a 4-well multidish (30 zygotes per well) with each well containing 500 µL of the same medium under mineral oil. The presumptive zygotes were cultured at 39°C in a humidified atmosphere of 5% CO$_2$ in air for 18 h to assess the fertilization parameters or for 7 days to assess embryo development. The presumptive zygotes were cultured in glucose-free NCSU-23 supplemented with 0.3 mM pyruvate and 4.5 mM lactate for the first 2 days and then in fresh NCSU-23 medium containing 5.5 mM glucose for an additional 5 days.
Assessment of maturation, sperm penetration and embryo development

To evaluate maturation and fertilization parameters, a random subset of the oocytes and presumptive zygotes was mounted on slides and fixes in 30% (v/v) acetic acid in ethanol for 48 to 72 h at room temperature. The oocytes were then stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope to assess the meiotic maturation. The maturation rate was assessed at 44 h of incubation. The oocytes were considered mature when their chromosomes were at MII and they had an extruded PB1. Fertilization parameters were evaluated 18 h after insemination. A randomly proportion of the presumptive zygotes were fixed and stained, as described above, to assess the fertilization parameters. The oocytes with a broken oolemma or abnormal appearance of the cytoplasm were deemed degenerated oocytes. The 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 criteria for pronuclei with normal morphology and chromatin configuration were based on pronuclei size and characteristics of the chromatin component inside the pronuclei (chromatin dispersion and staining intensity). The following fertilization parameters were evaluated: the penetration rate (number of oocytes penetrated/total inseminated), monospermy (number of oocytes containing only one male pronucleus/total penetrated), the number of spermatozoa/oocyte (mean number of spermatozoa in penetrated oocytes) and the efficiency of fertilization (number of monospermic oocytes/total inseminated oocytes).

At 2 and 7 days after insemination, the cleavage rate (percentage of embryos cleaved/total oocytes inseminated) and blastocyst formation (percentage of blastocysts/total oocytes inseminated), respectively, were evaluated under a stereomicroscope. An embryo that had cleaved to the 2-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 H342 (Sigma), followed by examination using fluorescence microscopy. The total number of nuclei that were stained with H342 and displayed blue fluorescence was counted.
Oocyte activation and in vitro culture

Cumulus-free in vitro-matured oocytes were placed in activation medium (0.3 M Mannitol, 1.0 mM CaCl2·H2O, 0.1 mM MgCl2·6H2O, and 0.5 mM Hepes) in a BTX microslide (Model #450-1) and were exposed to electrical pulses between 0.5 mm diameter stainless steel electrodes 1 mm apart. Activation was induced with two DC pulses of 1.2 kV/cm for 30 μs provided by a BTX Electro-Cell Manipulator ECM 2001 (BTX, San Diego, CA, USA). The activated oocytes were washed three times and transferred in groups of 30 - 40 into each well of a Nunc 4-well dish containing 500 μL of culture medium (NCSU-23 supplemented with 0.4% BSA) covered with mineral oil. Oocytes were cultured in 5% CO2 in air at 39°C for 7 days. Cleavage rate, total blastocyst rate, expanded blastocyst rate on day 7 and total cell numbers were assessed as described above.

Recovery of in vivo-derived oocytes, zygotes, 2- to 4-cell embryos and blastocysts

Animals

The experimental protocols were carried out in accordance with the Directive 2010/63/EU EEC for animal experiments and were reviewed and approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain. Weaned crossbred (Landrace x Large White) sows (from two to six parities) were randomly selected for the experiment. Sows were individually held in crates in a mechanically ventilated confinement facility under commercial production conditions (Agropor S.L, Murcia, Spain). Sows were fed a commercial ration twice a day, and water was provided ad libitum.

Detection of estrus and insemination

Estrus was detected twice a day, beginning 2 days after weaning, by exposing females to a mature boar and applying manual back-pressure. Females that showed a standing estrus reflex were considered to be in heat. Only sows that exhibited their first standing reflex on the afternoon of the 4th – 5th day after weaning were used in this experiment. Sows were artificially inseminated with doses of semen (3 x 10⁹ spermatozoa in 100 mL) from adult Pietrain boars extended with Beltsville Thawing Solution (BTS; Pursel and Johson, 1975) and kept for a maximum of 24 h at 17°C.
**Embryo collection medium**

The collection medium was Tyrode's lactate (TL)-Hepes PVA modified medium (Funahashi et al., 2000) supplemented with 10 mM Hepes (flushing medium).

**Collection of samples**

Sows were subjected to laparotomy, as described by Martinez et al. (2004). On Day 1 (Day 0 = onset of estrus) preovulatory follicles (> 8 mm in diameter) were aspirated using an 18-gauge needle connected to a 10-mL disposable syringe to obtain mature oocytes. On Day 2 of the estrous cycle, oviducts of each animal were flushed with 10 mL of flushing medium to obtain zygotes and 2- to 4-cell embryos. Blastocysts were collected on Day 6 of the estrous cycle by flushing the uterine horns with 30 mL of flushing medium. Oocytes and embryos were evaluated under a stereomicroscope at 60X to assess their developmental stage and quality. Only intact oocytes that had extruded the first polar body and fertilized oocytes with a single cell and two visible polar bodies were selected as mature oocytes and zygotes, respectively. Thereafter, oocytes and embryos were washed three times with flushing medium, placed in Eppendorf tubes containing 1.5 mL of the same medium and transported at 39°C in a thermostatically controlled incubator to our laboratory at the University of Murcia within 2 h of collection.

**Confocal microscopy of active mitochondria**

Mature oocytes, zygotes, 2- to 4-cell embryos and blastocysts in vivo or in vitro derived, were stained with MitoTracker Deep Red 633 (M22426; Molecular Probes, Leiden, the Netherlands) to assess the distribution of active mitochondria. A stock solution of the dye at a concentration of 1 mM was prepared in dimethyl sulfoxide and stored at -20°C. Oocytes and embryos were stained for active mitochondria in maturation or culture medium, respectively, containing 0.5 µM MitoTracker Deep Red for 30 min at 39°C in 5% CO₂ in air. Labeled oocytes and embryos were then washed in maturation or culture medium, respectively, three times for 20 min each at 39°C in 5% CO₂ in air. After washing, samples were fixed in 4% paraformaldehyde in a 0.1 M sodium cacodylate-buffered solution (pH range 7.2 to 7.4) for 2 h at room temperature. Fixed samples were washed three times for 5 min each in phosphate-buffered saline containing 3 mg/mL BSA. After the final wash,
oocytes and embryos were mounted on a slide in 4 µL Vectashield (Vector, Burlingame, CA, USA) containing 10 µg/mL H342 for nuclei staining. Samples were observed using a confocal laser scanning microscopy system (Leica TCS SP, Wetzlar, Germany). Fluorochromes were excited using appropriate combinations of excitation and barrier filters; an argon-krypton laser was used for the excitation of MitoTracker Deep Red (644 nm) and an ultraviolet laser was used for the excitation of H342 (354 nm). The images produced by sequential scanning were merged and recorded in a digital format. The images were subsequently displayed using Adobe Photoshop 7 (Adobe Systems, San Jose, CA, USA), and the distribution of active mitochondria was assessed.

**DNA extraction and real-time PCR quantification**

DNA was extracted from individual oocytes and embryos using the freeze–thaw method as previously described by Spikings et al. (2007). Briefly, oocytes, zygotes, 2- to 4-cell embryos and blastocysts were suspended individually in 50µL sterile nuclease-free water. Samples received two freeze-thaw cycles to lyse the oocytes/embryos and release DNA. To generate external standards a 296 bp fragment of mitochondrial DNA (mtDNA) (GeneBank accession number NC_012095.1) was amplified by polymerase chain reaction (PCR) as previously described (Spikings et al., 2007) using the following primers (forward primer: CTC AAC CCT AGC AGA AAC CA and reverse primer: TTA GTT GGT CGT ATC GGA ATC G). Then, the PCR product was run on 2% agarose gel and DNA was extracted from the excised bands using QiaQuick Gel Extraction kit (Qiagen, London) according to manufacturer’s instructions. The purified PCR product was then sequenced. Both primers and PCR product were verified for specificity using BLAST (http://blast.ncbi.nlm.nih.gov/). It was assumed that 21.73 ng of the 296 pb PCR product contains 6.79 x 10^10 double stranded DNA (PCR product length: 296 pb; Concentration: 21.73 ng/µL = 21.73 x 10^-9 g/µL; Calculation: (21.73 x 10^-9 g/µL / [296 x 660]) x 6.022 x 10^23 = 6.79 x 10^10 molecules/µL). These samples were serially diluted 10-fold in order to construct a standard curve for PCR quantification.

Quantitative Real-Time PCR (qPCR) analysis was performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The qPCR mixtures contained 12.5 µL 2X Power SYBR Green (Applied Biosystems, Warrington, UK), mtDNA primers at optimized concentrations (16.5 pM of each primer), 2 µL each sample (oocyte/embryos) or standard and made up to 25 µL with sterile nuclease-free water. The
reaction conditions were 1 cycle at 95°C for 10 min followed by 50 cycles of 94°C for 10 s, 
56°C for 15 s, and 72°C for 30 s. For each qPCR run, a standard curve was generated using five 10-fold serial dilutions (10^2 - 10^7 copies) of the target mtDNA PCR product with the same primers as those used for oocyte and embryos mtDNA amplification. The 7300 ABI system software generated a standard curve (plot of C\text{t} value/crossing point against log of amount of standard). The C\text{t} value of the target was compared with the standard curve, allowing calculation of the copy number of mtDNA in each sample. Dissociation curve was analysed to ensure the specificity of the amplified products. All reactions were run in duplicate and the mean quantity of mtDNA was calculated using the 7300 ABI system software on the base of the standard curve.

**Microtubule and chromosome immunostaining-confocal microscopy**

Oocytes were fixed in 4% (w/v) formaldehyde-PBS, permeabilized using Triton X-100 (2.5% (v/v) in PBS) for 15 min, and simultaneously immunostained for α-tubulin and chromatin detection (Morató et al., 2008). Fixation and subsequent incubations were performed at 37°C. For immunostaining, fixed oocytes were incubated with the anti-α-tubulin monoclonal antibody (mouse IgG1 isotype) (1:4000) for 90 min, followed by incubation with the goat antimouse IgG antibody-Alexa Fluor 488 (Molecular Probes-Invitrogen, UK) (1:500) for 1 h. Between incubations, the oocytes were washed three times in pre-warmed PBS for 5 min. Oocytes were individually mounted on coverslips fitted with a self-adhesive reinforcement ring and then stained with Vectashield – mounting medium for fluorescence with DAPI (1.5 μg/mL) (Vector Laboratories, Inc., Burlingame, CA, USA). The preparation was sealed with nail varnish and protected from light until observation. Negative controls for α-tubulin were produced using only the secondary antibody. A laser-scanning confocal microscope (Leica TCS-SP2-AOBS) was used to examine tubulin (Alexafluor 488; excitation 488 nm) and chromatin (DAPI; excitation 405 nm). Images were recorded on a computer. Spindle morphology and chromosomes were classified in three categories: 1) Normal spindle: barrelshaped with chromosomes clustered as a discrete bundle at the metaphase plate and microtubules crossing the length of the spindle from pole to pole. 2) Abnormal spindle: microtubules were not organized as typical spindles or some microtubules were disassembled with chromosomes with some degree of disarrangement or displacement from the metaphase plate. 3) Absence of the spindle: no microtubules could be observed around the chromosomes.
Spindle visualization by Polarized Light Microscopy

Oocytes were placed individually in 10 μL drops of TCM199-Hepes-FCS (TCM199-Invitrogen, Barcelona, Spain, 25 mM Hepes and 10% Fetal Calf Serum) covered with mineral oil in a glass Petri dish. Meiotic spindle visualization was performed using an inverted microscope (Olympus IX71, Japan) at 200X magnification with the Oosight system (CRI, Woburn, MA, USA) that consists of a liquid-crystal tunable filter optic, a circular polarizer/green interference filter optic, a scientific-grade CCD camera and appropriate software for image acquisition and analysis. During the whole evaluation, oocytes were maintained at 38°C on a heated stage (ThermoPlate-Tokai Hit, Japan). Each oocyte was rotated using two micromanipulators (Eppendorf, Germany) and micropipettes that help to hold and turn around the oocytes until the meiotic spindle was visualized as a white spot (positive signal) or classified as not having a positive signal.

EXPERIMENTAL DESING AND RESULTS

Objective 1: To evaluate the effects of exposure to H342 staining and UV irradiation on fertilisability and subsequent developmental capacity of porcine oocytes matured in vitro.

Experimental design

Experiment 1.1: Effect of H342 and ultraviolet irradiation for 30 s.

In this experiment, the individual and/or combined effects of H342 for 12 min and UV irradiation for 30 s on the developmental ability of in vitro-matured oocytes were evaluated. Immediately after maturation, groups of 30 oocytes were incubated for 12 min in 500 μL of maturation medium containing 0 or 5 μg/mL H342. Subsequently, the oocytes were washed in maturation medium and individually allocated into drops of 20 μL of maturation medium overlaid with warm mineral oil to be exposed or not exposed to UV irradiation, according to the experimental design. A total of 1388 in vitro-matured oocytes from five replicates were randomly allocated in the following groups: Group 1: oocytes without treatment (Control), Group 2: oocytes stained with H342, Group 3: oocytes stained with H342 and UV irradiated for 30 s, and Group 4: oocytes UV irradiated for 30 s. After the treatments, the oocytes from
each group were pooled, exposed to thawed spermatozoa and cultured for 18 h (n = 437) to assess fertilization parameters or for 7 days (n = 951) to evaluate embryo development.

Experiment 1.2: Effect of H342 and ultraviolet irradiation for 5 s.

Because the results from experiment 1.1 revealed that the exposure of H342-stained oocytes to UV irradiation for 30 s limited their developmental competence, this study was designed to evaluate the effect of a shorter UV irradiation (5 s) on fertilization parameters and on embryo development. A total of 1835 in vitro-matured oocytes from six replicates were separated into the same groups as Experiment 1.1. The only difference was that in the groups with UV irradiation, the oocytes were subjected to radiation for only 5 s. After the treatments, the oocytes were incubated with thawed spermatozoa and cultured for 18 h (n = 749) to assess fertilization parameters or for 7 days (n = 1086) to evaluate embryo development.

Results

Experiment 1.1.

The total oocyte maturation rate ranged from 75.2 ± 6.7% to 82.9 ± 6.4% among the replicates; the differences were not statistically significant. The effectiveness of the in vitro fertilization (IVF) procedure used in this study was similar to that obtained in other experiments in our laboratory, with more than 90% and 50% penetration and monospermy rates, respectively, and a final efficiency of approximately 44%. Sperm penetration (P < 0.001) and monospermy (P < 0.04) were lower in oocytes exposed to H342/UV (80.7 ± 4.5% and 30.7 ± 5.4%, respectively) than in oocytes from the Control group (94.9 ± 4.3% and 50.0 ± 4.9%, respectively), and consequently, the efficiency of fertilization was also lower (P < 0.04). In each group, all oocytes fertilized formed a female pronucleus with a normal morphology and chromatin configuration. Normal male pronuclei were formed in more than 97% of the oocytes fertilized, irrespective of the treatment used. In addition to male pronuclei, decondensed sperm heads within the cytoplasm were presented in less than 8% of the oocytes, with no differences among the groups. In these oocytes, the number of decondensed sperm heads per oocyte ranged from 1 to 3. The oocytes exposed to H342/UV showed a lower (P < 0.001) cleavage (49.8 ± 2.9%) and blastocyst (7.7 ± 2.9%) rate than
oocytes from the other groups (range: 73.8 ± 2.9% to 77.7 ± 2.9% and 22.3 ± 2.9% to 30.9 ± 3.0%, respectively). No differences were observed in the blastocyst total cell number among the groups.

Experiment 1.2.

There were no significant differences in the maturation rates among the replicates (range: 71.6 ± 6.4% to 78.8 ± 7.2%). Irrespective of the treatment, normal female and male pronuclear formation was seen in more than 96% of the oocytes fertilized, and the percentage of oocytes with additional sperm heads was lower than 6%. Penetration and monospermy rates, the number of spermatozoa per oocyte penetrated, the final efficiency of IVF and the cleavage rates were not influenced by the different treatments. However, oocytes exposed to H342 and UV irradiation for 5 s showed a lower (P < 0.02) rate of blastocyst formation (15.2 ± 4.5%) than oocytes from the other groups (range: 26.1 ± 4.5% to 30.7 ± 4.5%). No differences were observed in the blastocyst total cell number among the groups.

These results show that exposure of the oocytes to H342 in combination with UV irradiation has a deleterious effect on developmental competence of in vitro-matured porcine even for period as short as 5 s.

Objective 2: To evaluate the effects of H342 staining and UV irradiation on mitochondrial distribution and mtDNA copy number in developing porcine oocytes and pre-implantation embryos.

Experimental design

In this study, the distribution of active mitochondria and the quantification of mtDNA copies were evaluated in in vitro- and in vivo-derived mature oocytes and embryos. A total of 1426 in vitro-matured oocytes from three replicates were exposed to H342 and UV for 30 s (H342/UV) or non-exposed (in vitro Control group), and subjected to IVF with frozen-thawed spermatozoa and in vitro culture. In each replicate, a random subset of the in vitro matured oocytes, zygotes, 2- to 4-cell embryos and blastocysts were used to
determine the distribution of active mitochondria and the mtDNA copy number and the remaining were used to evaluate embryo development. Only intact oocytes that had extruded the first polar body and fertilized oocytes with a single cell and two visible polar bodies were selected as mature oocytes and zygotes, respectively. In vivo-derived oocytes and embryos (n=150) were collected from 10 sows in two replicates (2-3 sows/type of sample) and used as controls. For mitochondrial distribution, all samples (oocytes and embryos) were stained and fixed until confocal evaluation. For mtDNA copy number the samples were suspended individually in 50 µL nuclease-free water and frozen at -80°C until assay. The qPCR was run in 96-well plates in a total of two replicates. To avoid variation, each plate contained oocytes and embryos from each source and developmental stage.

**Results**

*Effect of H342 and UV irradiation on the development of in vitro-fertilized oocytes.*

A total of 1075 in vitro-matured oocytes were inseminated and cultured for 7 days to evaluate embryo development. The oocytes exposed to H342/UV showed a lower (P < 0.001) cleavage rate (40.9 ± 12.2%) and blastocyst formation (10.6 ± 1.6%) than oocytes from the Control group (62.4 ± 12.2% and 24.4 ± 1.6%, respectively). No differences were observed in blastocyst total cell number between groups.

*Effects of H342 and UV irradiation on the distribution of active mitochondria in mature oocytes and preimplantation embryos.*

At collection, in most (11/13; 84.6%) of the in vivo-matured oocytes, active mitochondria were distributed throughout the cytoplasm and were more abundant in the peripheral cytoplasm than in the inner cytoplasm. Strong active mitochondrial staining was commonly observed in the first polar body. This distribution of active mitochondria was also present in 90.9% of the in vitro Control matured oocytes and in 68.4% of the in vitro-matured oocytes exposed to H342/UV (20/22 and 13/19, respectively). Oocytes with poor or no staining for mitochondria were considered to be mature oocytes with abnormal mitochondrial distribution. After fertilization, 100% of the in vivo zygotes (18/18) showed active mitochondrial aggregations associated with the pronuclei in the inner cytoplasm and near the plasma membrane. There was also strong mitochondrial staining in the first and
second polar bodies. This pattern was observed in 75% (6/8) and 58.3% (7/12) of the in vitro monospermic zygote controls and those exposed to H342/UV, respectively. In vitro zygotes with no mitochondria around of the pronuclei were considered to be zygotes with abnormal mitochondrial distribution. During early cleavage, in most of in vivo (25/26; 96.2%) and in vitro Control (24/27; 88.9%) 2- to 4-cell embryos, active mitochondria were distributed homogenously in the inner cytoplasm mainly around the periphery of each blastomere, and aggregated in the nuclear region. In contrast with the 2- to 4-cell in vivo and in vitro Control embryos, only 13/30 (43.3%) of the 2- to 4-cell embryos obtained from the oocytes exposed to H342/UV showed that distribution. Additionally, 20% (6/30) of the embryos in the exposed group showed active mitochondria with strong staining in anucleate cell fragments, and 36.6% (11/30) presented blastomeres with poor or no staining for mitochondria. Active mitochondria were distributed homogeneously in both the inner cell mass and trophectoderm cells in all blastocysts analyzed, irrespective of the experimental group.

**Effects of H342 and UV irradiation on mtDNA copy number in mature oocytes and preimplantation embryos.**

There were no significant differences in the mtDNA copy number among in vivo, in vitro Control and in vitro exposed mature oocytes (range: 347023 ± 46334 to 400615 ± 89487). However, the mtDNA copy numbers of the embryos obtained from the oocytes exposed to H342/UV were lower (P < 0.04) for zygotes (85909 ± 19503) and 2- to 4-cell embryos (110280 ± 23082) and higher (P < 0.02) for blastocysts (557115 ± 176177) than those obtained from in vivo-derived embryos (162324 ± 25508; 180878 ± 17009; and 167287 ± 33379, respectively). No differences were observed among in vivo and in vitro Control oocytes and embryos. When comparisons were made within each experimental group (in vivo or in vitro, exposed or non-exposed to H342/UV), significant differences were found according to developmental stage. In the in vivo group, the mature oocytes contained an average of 400615 ± 89487 mtDNA copies, which was higher (P < 0.02) than in zygotes, 2- to 4-cell embryos and blastocysts (162324 ± 25508; 180878 ± 17009; and 167287 ± 33379, respectively). In the in vitro groups, the average mtDNA copy number was 347023 ± 46334 and 370441 ± 77126 for in vitro Control oocytes and oocytes exposed to H342/UV, respectively; this value decreased (P < 0.02) in zygotes (100575 ± 20895 and 176192 ± 26607, respectively) and 2- to 4-cell embryos (85909 ± 19503 and 110280 ± 23082, respectively).
Nevertheless, mtDNA copy number in blastocysts increased to $320056 \pm 52590$ and $557115 \pm 176717$ for the Control and H342/UV groups, respectively, and these numbers did not differ significantly from those of mature oocytes.

In the present study it is shown that the simultaneous exposure of mature in vitro porcine oocytes to H342 staining and UV irradiation is associated with reduced oocyte developmental competence, and abnormal mitochondrial distribution pattern and reduced mtDNA copy number in the resulting cleaved embryos. Such outcomes have to be considered when H342 and UV irradiation are used to aid or confirm enucleation for nuclear transfer.

**Objective 3: To investigate the effectiveness of a longer-wavelength fluorochrome (SYBR-14) for visualizing maternal chromosomes in in vitro-matured porcine oocytes and the effects of this dye in combination with fluorescence excitation on the subsequent in vitro fertilization and embryo development of the oocytes.**

**Experimental design**

**Experiment 3.1: Visualization of the MII plate and the PB1 of in vitro-matured oocytes exposed to different concentrations of SYBR-14 and incubation times.**

To determine the optimal conditions for enucleation, this experiment was conducted to evaluate the effect of different concentrations of SYBR-14 (Invitrogen, Eugene, OR, USA) and times of incubation on the visualization of the chromosomes at MII and the PB1 in the mature oocytes. Immediately after maturation, a total of 913 denuded oocytes from 3 replicates were incubated in 100 μL of maturation medium at different concentrations (1, 3, 5 and 7 μg/mL) of SYBR-14 and incubation times (5, 10 and 30 min) in a 4 x 3 experimental design. Next, the oocytes were washed in maturation medium and individually allocated into drops of 20 μL of maturation medium overlaid with warm mineral oil to be exposed to fluorescence. The MII plate and PB1 were visualized by excitation with a fluorescence microscope (Nikon Eclipse TE2000-S®). The fluorescence was observed using a 485-nm excitation line and a 520-nm emission filter. The oocytes were considered correctly stained when both the MII plate and the PB1 were visualized. To confirm the
results of visualization, all the oocytes were fixed in acetic acid/ethanol, stained with lacmoid and examined under a phase-contrast microscope, as described above. The efficiency of visualization was defined as the number of oocytes with MII plate and PB1 visualized using live fluorescence evaluation divided by the number of oocytes with MII plate and PB1 visualized using fixation and staining. The optimal concentration and incubation time considered in this experiment were checked to evaluate the efficiency of enucleation. After 44 h of maturation, 86 denuded oocytes were incubated for 10 min in maturation medium containing 5 μg/mL SYBR-14. The oocytes were loaded individually into 20-μL droplets overlaid with mineral oil. Each oocyte was rotated with the help of two micromanipulators (Eppendorf, Germany) to set the MII plate at a 3 o’clock position. The MII chromosomes and the PB1 were aspirated using a 17-μm bevelled glass pipette. To confirm successful enucleation, all the enucleated oocytes were evaluated by mounting each oocyte on a slide in 4 μL of a dilution of glycerol-DPBS (3:1) containing 10 μg/mL H342, followed by examination using fluorescence microscopy. The efficiency of enucleation was defined as the number of oocytes without residual DNA after enucleation divided by the total number of oocytes subjected to enucleation.

Experiment 3.2: Effect of SYBR-14 and exposure to fluorescence on the developmental ability of in vitro–matured oocytes exposed to fluorescence.

This study was designed to evaluate the effects of SYBR-14 staining and fluorescence excitation for 5 or 30 s on the developmental ability of in vitro–matured oocytes. The best conditions determined from the first experiment was incubation with 5 μg/mL SYBR-14 for 10 min. Immediately after maturation, groups of 30 oocytes were incubated for 10 min in 100 μL of maturation medium containing 0 or 5 μg/mL SYBR-14. Next, the oocytes were washed in maturation medium and individually placed in drops of 20 μL of maturation medium overlaid with warm mineral oil to be exposed or not exposed to fluorescence, according to the experimental design.

A total of 1708 in vitro-matured oocytes from five replicates were randomly allocated into the following groups: Group 1: untreated oocytes (Control), Group 2: oocytes exposed to SYBR-14 staining, Group 3: oocytes exposed to SYBR-14 staining and fluorescence for 5 s and Group 4: oocytes exposed to SYBR-14 staining and fluorescence for 30 s. After the treatments, the oocytes from each group were pooled, exposed to thawed
spermatozoa for 6 h and cultured for 12 h (n=510) to assess the fertilization parameters or cultured for 7 days (n=1198) to evaluate the embryo development.

In an additional trial conducted as part of this study, the fertilization parameters and the developmental ability of oocytes exposed (5 s or 30 s) or not exposed to fluorescence light were evaluated. We used the same method described above except that the oocytes were not incubated with SYBR-14. A total of 782 in vitro-matured oocytes from three replicates were randomly allocated into the following groups: Group 1: untreated oocytes (Control), Group 2: oocytes exposed to fluorescence for 5 s and Group 3: oocytes exposed to fluorescence for 30 s. After treatments, the oocytes were inseminated and cultured for 12 h (n=280) or for 7 days (n=502) as described above.

Results

Experiment 3.1.

The analysis of variance showed that the dye concentration, the incubation time and the interactions of both factors influenced the percentages of MII plate and PB1 that could be properly visualized. The efficiency of visualization was highest with a dye concentration of 5 μg/mL and an exposure time of 10 min or longer or with a dye concentration of 7 μg/mL and an exposure time of 5 min or longer. No increase in the intensity of the stain was detected visually after 10 min or with the highest SYBR-14 concentration. To use the minimum concentration of stain required for the visualization of the MII plate and PB1, 5 μg/mL of SYBR-14 and 10 min of incubation were chosen for the subsequent experiment. In that group, the efficiency of visualization was 81.3 ± 4.9%. In addition, the fluorescence remained observable after 40 min, and 82 out of 86 oocytes were properly enucleated with an efficiency of enucleation of 95.3%.

Experiment 3.2.

The mean oocyte maturation rate after 44 h of incubation was 80.7 ± 6.1%; the differences among the replicates were not statistically significant. At 18 h after exposure, the degeneration rates of the oocytes exposed to SYBR-14 (5 μg/mL for 10 min) and fluorescence were significantly higher (P < 0.002) than those for the other groups. To
evaluate the specific effects of SYBR-14 and fluorescent light on the fertilization parameters. Degenerated and immature oocytes were excluded from the analysis, and only mature oocytes were considered. The penetration (range: 66.7 ± 6.1% to 76.9 ± 3.6%) and monospermy rates (range: 55.7 ± 4.9% to 58.9 ± 6.6%), the number of spermatozoa per oocyte penetrated (range: 1.6 ± 0.1 to 1.8 ± 0.1) and the final efficiency of fertilization (range: 38.3 ± 6.3% to 43.9 ± 4.2%) were not influenced by the different treatments. Exposure to only SYBR-14 did not adversely affect the in vitro development of the oocytes to the 2- to 4-cell and blastocyst stages. The cleavage and blastocyst rates were lower (P < 0.001) for the oocytes exposed to the SYBR-14 staining and the fluorescence for 5 s (31.5 ± 2.7% and 7.4 ± 1.5%, respectively) and 30 s (14.9 ± 2.1% and 2.0 ± 0.1%, respectively) than for those in the Control group (66.2 ± 2.7% and 27.8 ± 2.6%, respectively). The SYBR-14 and the fluorescence excitation for 5 and 30 s did decrease (P < 0.02) the proportion of cleaved embryos reaching the blastocyst stage in 18.6 and 30.1 points, respectively, compared with the Control group. No differences were observed in the blastocyst total cell number among the groups. Results obtained in the additional trial showed that the exposition of mature oocytes to only fluorescence illumination does not affect the rates of degeneration (range: 0.0 - 2.3%), fertilization and embryo development.

Our results demonstrate that the combination of SYBR-14 staining and fluorescence exposure for periods as short as 5 s exerts a clear deleterious effect on the viability and developmental ability of porcine oocytes.

**Objective 4:** To assess the efficiency of PLM to detect microtubule-polymerized protein and to assist in the removal of the meiotic spindle of *in vitro*-matured porcine oocytes and to examine the effects of PLM on the oocyte developmental competence.

**Experimental design**

*Experiment 4.1: Efficiency of PLM to detect microtubule-polymerized protein in *in vitro*-matured porcine oocytes.*

Oocytes were assessed by polarized light microscopy to detect the presence of microtubule-polymerized protein, which could be forming a meiotic spindle. A total of 117
oocytes from 7 replicates were assessed. The presence of polymerized protein and a meiotic spindle was confirmed in individual oocytes by immunostaining and chromatin detection. The positive signal detected in each oocyte by the PLM was assessed with the Oosight software to get the retardance value as a putative predictor of microtubule density and conformation.

Experiment 4.2: Effects of PLM on the oocyte developmental competence.

A total of 160 MII oocytes for each experimental group from 4 replicates were exposed or not (Control) to PLM for 10 min. Groups of 10 oocytes were exposed to PLM in 10 μL droplets. Thereafter, the oocytes were parthenogenetically activated and cultured in vitro.

Experiment 4.3: Selection of oocytes with PLM and evaluation of further developmental competence.

Oocytes were exposed to PLM and assessed to detect the presence or the absence of the meiotic spindle. Control oocytes were not exposed to PLM, so that meiotic spindle was not assessed. Oocytes being PLM positive were handled separately from their PLM negative counterparts. Oocytes from the three experimental groups (Control, PLM positive, PLM negative) were fertilized and cultured.

Experiment 4.4: Efficiency of PLM to assist the enucleation of MII oocytes.

MII oocytes were stained with 1μg/mL DNA dye H342 and were loaded individually into 20 μL microdroplets. Each oocyte was rotated with the help of two micromanipulators to set the meiotic spindle at 3 o’clock position, followed by aspiration of the second meiotic spindle under polarized light microscopy. Successful removal of the meiotic spindle and chromosomes were confirmed by exposing all the removed cytoplasm, to both the PLM-Oosight system and checking for the presence of the removed spindle and to UV light to confirm complete removal of the maternal chromosomes. The experiment was replicated three times.
**Results**

*Experiment 4.1.*

A positive PLM signal was detected in 98.2% of the oocytes and 96.5% of them reached the MII stage. All of MII oocytes, but one, were positive to PLM. There was a positive correlation ($r = 1; P < 0.0001$) between the signal obtained by PLM and the presence of microtubule-polymerized protein as confirmed by immunostaining. A barrelshape spindle was observed in 92.9% of the MII oocytes by immunostaining and an abnormal meiotic spindle conformation was detected in 6.2% of MII oocytes. In addition, the retardance values obtained from in vitro-matured porcine oocytes after being exposed to PLM ranged from 1.87 to 6.38 nm and the mean retardance value of oocytes with normal spindle (barrel shape spindle) ($3.9 \pm 0.1$ nm) did not differ from oocytes with abnormal spindle ($3.2 \pm 0.4$ nm) assessed by immunostaining.

*Experiment 4.2.*

After been assessed by PLM, oocytes were pooled together and parthenogenetically activated and cultured in vitro. Oocytes exposed to PLM did not differ significantly from controls in cleavage rate (83.7 ± 1.5% and 84.4 ± 1.5%, respectively), total blastocyst rate (36.9 ± 3.6% and 41.2 ± 3.6%, respectively) and expanded blastocyst rate on day 7 (21.9 ± 1.7% and 26.2 ± 1.7%, respectively). There were also no differences in total cell numbers counted in expanded blastocysts.

*Experiment 4.3.*

In spite of the well-known differences between males in IVF results, in this study frozen sperm from a well characterized boar was used to fertilize the oocytes instead of parthenogenetically activating them. The objective was to obtain embryos with the potential to produce live offspring. The penetration rates (range: 66.7 ± 4.8% to 72.2 ± 4.8%), monospermy rate (range: 60.6 ± 6.2% to 66.4 ± 6.2%) and the efficiency of fertilization (36.5 ± 3.8% to 42.4 ± 3.8%) did not differ significantly among groups. However, the percentage of oocytes that reached the MII stage in the negative PLM group (80.2 ± 1.6%) significantly differed from the Control and PLM positive groups (96.0 ± 1.6% and 100.0 ± 1.6%, respectively).
respectively). There were no significant differences among groups in cleavage rate (range: 53.1 ± 7.6% to 70.5 ± 5.7%). However, significant differences in blastocyst formation rate were observed between the PLM negative group (11.5 ± 2.4%) and the other groups (Control, 25.5 ± 1.8%; PLM positive, 27.7 ± 1.8%). Total cell numbers at blastocyst stage did not differ among Control, PLM positive and PLM negative groups.

Experiment 4.4.

Fifty-four MII oocytes were enucleated under the Oosight system with an overall efficiency of 92.6%. The procedure was simple and a very small amount of cytoplasm was removed in each enucleation.

These results show that PLM is an efficient system to detect microtubule-polymerized protein in in vitro-matured porcine oocytes. Moreover, this procedure is an efficient technique to remove meiotic spindle without to exert detrimental effects on pig oocytes developmental competence and can be used as a tool to improve some reproductive technologies such as nuclear transfer.
CONCLUSIONS
CONCLUSIONS

1. The exposure of porcine oocytes to Hoechst 33342 staining or ultraviolet irradiation, separately, does not interfere with their fertilization and developmental potential. However, Hoechst 33342 combined with ultraviolet irradiation has deleterious effects on the development competence of the oocytes, with the effects being more drastic with increased exposure to UV irradiation.

2. The exposure of the oocytes to Hoechst 33342 staining and ultraviolet irradiation is associated with abnormal mitochondrial distribution pattern and with reduced mitochondrial DNA copy number in the resulting cleaved embryos. Such outcomes have to be considered when Hoechst 33342 and ultraviolet irradiation are used to aid or confirm enucleation for nuclear transfer.

3. The combination of SYBR-14 staining and fluorescence for periods as short as 5 s exerts a clear deleterious effect on the viability and developmental ability of porcine oocytes. These results indicate that SYBR-14 staining should be avoided for enucleation purposes in pigs.

4. Polarized Light Microscopy is an efficient system to detect microtubule-polymerized protein in in vitro-matured porcine oocytes with no detrimental effect on developmental ability of in vitro porcine oocyte. This technique might to be an efficient method to enucleate porcine oocytes, susceptible of being used within the whole process of nuclear transfer in pigs.
RESUMEN GENERAL
INTRODUCCIÓN

Durante los últimos años, la transferencia nuclear de células somáticas (SCNT) ha sido ampliamente utilizada para la clonación de diversas especies de mamíferos, como bovino, ratón, cabra, porcino, gato, conejo, caballo, rata, perro y hurón (revisión de Meissner y Jaenisch, 2006; Campbell y cols., 2007). En concreto, la clonación en porcino por SCNT se ha convertido en una herramienta muy útil para la elaboración de modelos genéticos de enfermedades humanas y para el uso de los xenotransplantes (Dai y cols., 2002; Lai y cols., 2002; Fujimura y cols., 2008; Rogers y cols., 2008; Yazaki y cols., 2009). A pesar de que el número de lechones clonados obtenidos a través de células somáticas ha ido aumentando tras su primera publicación en el año 2000 (Betthauser et al., 2000; Onishi et al., 2000; Polaejaeva et al., 2000), la eficiencia total de esta tecnología aún permanece baja (reviewed in Vajta et al., 2007).

La metodología para realizar la transferencia nuclear conlleva numerosos pasos y puede verse afectada por diversos factores, como por ejemplo, el estado y el tipo de célula donante, la fuente y la calidad de los ovocitos, la preparación y la manipulación del citoplasma recipiente, la sincronización y los métodos de manipulación, la activación y finalmente, las condiciones de cultivo embrionario, cada uno de cuales, pueden llegar a influir sobre la eficiencia de esta técnica.

Uno de los pasos más difíciles de la técnica de SCNT en porcino, es la enucleación de los ovocitos porque sus citoplasmas contienen numerosas gotas de lípidos (Genicot y cols., 2005) que imposibilitan la visualización de los cromosomas de los ovocitos mediante un microscopio estándar.

Aunque han sido descritos numerosos métodos para enucelar ovocitos porcinos, la tinción con el fluorocromo Hoechst 33342 (H342) con la posterior irradiación con luz ultravioleta (UV) es la técnica más frecuentemente utilizada para ayudar a la localización y a la visualización de la placa metáfásica y del primer corpúsculo polar (PB1) antes de la enucleación (Estrada y cols., 2008; Lee y cols., 2008; Hickley y cols., 2011) o para confirmar
dicha enucleación de los ovocitos después de la manipulación (Kragh y cols., 2005; Das y cols., 2010; Koo y cols., 2010; Biswas y cols., 2011).

El H342 es un fluorocromo vital permeable a la membrana plasmática, que emite fluorescencia azul cuando se excita con luz UV (350 nm). Esta tinción es ampliamente utilizada para teñir la cromatina de los ovocitos ya que el H342 es capaz de unirse al surco menor del DNA (Robison y cols., 1998).

A pesar de que se han obtenido lechones vivos a partir de ovocitos enucleados mediante la tinción con H342 y posterior exposición a la luz UV, se asume que la exposición de los ovocitos al H342 y a la luz UV podría tener un efecto perjudicial sobre su DNA y sobre sus orgánulos. Sin embargo, el efecto exacto de la exposición a H342 y de la irradiación con UV sobre los ovocitos aún no es del todo conocida, ya que sólo existen unos pocos estudios en determinadas especies como bovino, cabra, conejo y ratón (Tsunoda y cols., 1988; Yang y cols., 1990; Westhusin y cols., 1992; Smith y cols., 1993; Dominko y cols., 2000; Velilla y cols., 2002; Versieren y cols., 2010) y además, estos estudios muestran resultados variables dependiendo de la especie y del tiempo de exposición. A pesar del uso tan extendido del H342 en combinación con la luz UV para enuclear los ovocitos porcinos, hasta la fecha no existen estudios que reflejen los efectos concretos de esta técnica sobre la capacidad de desarrollo de los ovocitos porcinos.

Si la tinción con H342 y la irradiación con UV pueden tener un efecto perjudicial sobre la capacidad de desarrollo de los ovocitos, podría ser importante encontrar un método alternativo para visualizar los cromosomas maternos de los ovocitos maduros con el fin de facilitar su enucleación con la menor cantidad de citoplasma posible, conservando el volumen del ovocito sin comprometer la eficiencia de la enucleación y por lo tanto su capacidad de desarrollo. En este sentido, Dominko y cols. (2000), indicaron que la tinción con SYBR-14, fluorocromo de longitud de onda larga (485 nm), que tiñe el ácido nucleico y es permeable a las membranas, podría ser una alternativa al H342 porque la exposición al SYBR-14 no comprometió la capacidad de desarrollo de ovocitos bovinos tras su activación partenogenética. El uso de fluorocromos de longitud de onda larga podría permitir la visualización de los cromosomas maternos con una transferencia de energía menor resultando menos perjudicial a los ovocitos. Sin embargo, hasta la fecha no hay ningún
estudio que haya investigado los efectos de este método alternativo en otras especies, incluida en la especie porcina.

El Microscopio de Luz Polarizada (PLM), también llamado “Spindle View System”, podría ser otra posible alternativa para la visualización de los cromosomas maternos de ovocitos porcinos. Los microtúbulos del huso meiótico en los ovocitos maduros, son birrefringentes a la luz polarizada, creándose un contraste entre el huso y el resto de la célula. Comparado con la mayoría de los métodos de evaluación del huso meiótico que requieren la fijación y tinción de los microtúbulos de los ovocitos, el PLM ofrece una ventaja única al no ser invasivo, conservando la viabilidad del ovocito y permitiendo repetir las observaciones de la muestra.

Los principios y el equipo del PLM ha sido extensamente descrito por diversos autores (Oldenbourg y cols., 1996; Wang y cols., 2001; Keefe y cols., 2005; Shen y cols., 2008; Caamaño y cols., 2010). La detección del huso meiótico mediante esta técnica, parece estar asociado con un incremento en los porcentajes de fertilización y de la calidad embrionaria en ratón y en humana (Wang y cols., 2001, 2002; Coticchio y cols., 2004). Curiosamente, un mayor número de ovocitos con husos mitóticos detectados con PLM fueron fertilizados y alcanzaron el estadio de blastocisto, en comparación con aquellos ovocitos con husos mitóticos no detectados (Raju et al., 2007).

Además, se ha propuesto el valor de retardo (un indicador de la densidad de los microtúbulos) como un marcador de la calidad del ovocito en humana, y por lo tanto podría ser utilizado para seleccionar ovocitos con el fin de obtener una mejora en la capacidad de desarrollo embrionario (Liu y cols., 2000; De Santis y cols., 2005; Kilani y cols., 2009).

La estructura y la conformación de los husos meióticos son importantes para predecir la calidad y la viabilidad de los ovocitos. Si el PLM es capaz de detectar las anormalidades en el huso podría convertirse en una herramienta muy importante para seleccionar ovocitos con husos meióticos con morfología normal y con un alto potencial de capacidad de desarrollo. Las investigaciones con PLM se han llevado a cabo sobre todo con ovocitos de ratón y de humana. Sin embargo, la información sobre el uso práctico de PLM es muy limitada en ovocitos de animales de granja, como en cerdos, y sus posibles aplicaciones en la reproducción animal necesita estudios adicionales.
Basándonos en todo lo anteriormente expuesto, el presente trabajo fue diseñado con el propósito de estudiar los efectos de diversas técnicas para visualizar la placa metafásica y el primer corpúsculo polar sobre la capacidad de desarrollo de ovocitos porcinos madurados in vitro.
OBJETIVOS

El objetivo principal de esta tesis fue evaluar los efectos de diversas técnicas para visualizar la placa metafásica y el corpúsculo polar sobre ovocitos porcinos fecundados in vitro y su posterior desarrollo embrionario. Con este propósito, los objetivos específicos que contienen esta tesis fueron los siguientes:

1. Evaluar los efectos de la exposición a la tinción con Hoechst 33342 y a la luz ultravioleta, sobre la fertilidad y posterior capacidad de desarrollo de los ovocitos porcinos madurados in vitro. (Artículo 1).

2. Evaluar los efectos de la tinción con Hoechst 33342 en combinación con la luz ultravioleta sobre la distribución mitocondrial y el número de copias de DNA mitocondrial de los ovocitos y de los embriones preimplantacionales porcinos. (Artículo 2).

3. Investigar la efectividad de un fluorocromo de longitud de onda larga (SYBR-14) para visualizar los cromosomas maternos de los ovocitos porcinos madurados in vitro y los efectos de esta tinción en combinación con la excitación de la fluorescencia sobre la fecundación in vitro y posterior desarrollo embrionario de los ovocitos. (Artículo 3).

4. Evaluar la eficiencia de Microscopio de Luz Polarizada para detectar y asistir la retirada del huso meiótico de los ovocitos porcinos madurados in vitro, y para determinar su efecto sobre el desarrollo embrionario de los ovocitos. (Artículo 4).
MATERIAL Y MÉTODOS

Maduración y fecundación in vitro de ovocitos porcinos, y cultivo in vitro de embriones tempranos

Recolección y cultivo de los complejos cúmulos – ovocitos

Los ovocitos utilizados para la realización de la presente tesis se obtuvieron a partir de ovarios procedentes de cerdas prepuberales sacrificadas en un matadero industrial. Los ovarios fueron transportados al laboratorio a 35°C en una solución salina (0’9% NaCl) con Kanamicina (70 µg/mL), en la hora posterior de su recolección. Los complejos cúmulos-ovocitos (COCs) fueron obtenidos por aspiración de folículos antrales de tamaño medio (3 a 6 mm) de diámetro usando una aguja de 18 g conectada a una jeringa de 10 mL. Para la recolección y lavado de COCs fue usada una solución salina fosfatada de Dulbecco (DPBS, Dulbecco’s phosphate-buffered saline), compuesto por 136’9 mM NaCl, 2’7 mM KCl, 8’1 mM Na₂HPO₄ y 1’5 mM CaCl₂·2H₂O, suplementada con 4 mg/mL albúmina sérica bovina (BSA; Sigma-Aldrich Quimica SA, Madrid, Spain), 0’3 mM piruvato sódico, 5 mM D-glucosa and 70 µg/mL kanamicina (mDPBS). Se seleccionaron aquellos ovocitos que presentaban varias capas de células del cúmulo, y citoplasma uniformemente granulado. Los ovocitos seleccionados fueron lavados 3 veces en medio de maduración. El medio de maduración de los ovocitos fue NCSU-23 (North Carolina State University medium; Petter y Wells, 1993) libre de BSA, suplementado con 10% (v/v) fluido folicular porcino (FF), 0’8 mM cisteína, 10 ng/mL factor de crecimiento epidérmico and 5 nM 9-cis ácido retinoico. El FF porcino fue obtenido por aspiración de folículos entre 3 y 6 mm de ovarios procedentes de matadero como ha sido descrito anteriormente, fue centrifugado a 1500 x g durante 30 min a 15°C, el sobrenadante resultante fue filtrado a través de filtros de 0’8, 0’45 y 0’22 µm, alicuotado y almacenado a -20°C hasta su utilización. La maduración se llevó a cabo en placas multidish de 4 pocillos (Nunc, Roskilde, Denmark) en grupos de 70 a 80 COCs en 500 µL de medio de maduración suplementado con 10 IU/mL de gonadotropina coriónica equina (Folligon, Intervet International B.V., Boxmeer, the Netherlands) y 10 IU/mL de gonadotropina coriónica humana (Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain) durante las primeras 20-22 h, y posteriormente en medio de maduración sin hormonas las siguientes 20-22 h. Los pocillos estaban cubiertos con aceite mineral y la maduración se realizó en una
atmósfera de 5% de CO₂ en aire, a 39°C y con una humedad relativa del 95-100%. Tras la maduración, COCs fueron denudados con 0’1% de hialuronidasa en medio de maduración mediante vortex durante 2 min a 1.660 vueltas/min y a continuación, los ovocitos fueron lavados tres veces en medio de maduración.

**Fecundación y cultivo in vitro**

Una vez denudados, los ovocitos fueron lavados tres veces en medio de fecundación previamente equilibrado en el incubador. El medio de fecundación fue modified Tris-buffered medium (mTBM), compuesto por 113’1 mM NaCl, 3 mM KCl, 7’5 mM CaCl₂·2H₂O, 20 mM Tris, 11 mM glucosa and 5 mM piruvato sódico suplementado con 0’5 mM cafeína y 0’2% BSA (Abeydeera y Day, 1997). La fecundación in vitro fue realizada como describen Gil y cols. (2003); grupos de 30 ovocitos denudados fueron distribuidos en gotas de 50 µL de medio de fecundación en placas Petri de 35x10 mm (Falcon, Becton Dickinson Labware, Franklin Lakes, USA). Las gotas fueron cubiertas con aceite mineral y mantenidas en el incubador (5% CO₂ en aire, 39°C), durante aproximadamente 30 min hasta la adición de los espermatozoides.

El semen procedente de un verraco Pietrain fue procesado y criopreservado en pajuelas de 0’5 mL basándose en el método descrito por Carvajal y cols. (2004). Para cada replicado, se utilizó un pool de semen procedente de dos pajuelas descongeladas en un baño a 37°C durante 20 seg. Posteriormente, 100 µL del pool del semen descongelado fue lavados tres veces mediante centrífugación a 1.900 x g durante 3 min en mDPBS. El pellet resultante fue resuspendido en mTBM, de manera que cada 50 µL de esta suspensión espermática contenía 30.000 espermatozoides, para ser posteriormente añadido a la gota de fertilización que contenía los ovocitos. El ratio espermatozoide:ovocito fue 1000:1. Los gametos fueron coincubados bajo una atmosfera de 5% CO₂, a 39°C y con una humedad relativa de 95-100% durante 6 h.

Tras la fecundación in vitro, los cigotos fueron lavados tres veces en medio preequilibrado de cultivo de embriones (NCSU-23 con 0’4% BSA) y a continuación, transferidos a placas multidish de 4 pocillos en grupos de 30 cigotos por pocillo que contenían cada uno 500 µL del mismo medio cubierto de aceite mineral.
Los cigotos fueron cultivados bajo una atmósfera de 5% CO$_2$, a 39°C y humedad relativa de 95-100% durante 18 h para evaluar los parámetros de fertilización y durante 7 días para evaluar el desarrollo embrionario. Los embriones se cultivaron en NCSU-23 libre de glucosa suplementado con 0’3 mM piruvato y 4’5 mM lactato durante las primeras 48 h, y transcurrido ese tiempo el medio fue reemplazado por NCSU-23 con glucosa hasta el final del cultivo.

**Análisis de los parámetros de maduración, penetración espermática y desarrollo embrionario**

Para evaluar los parámetros de maduración y fecundación, un grupo al azar de ovocitos y de cigotos fueron montados sobre portaobjetos y fijados en una solución de acético:etanol 30% (v/v) durante 48-72 h a temperatura ambiente. Posteriormente, los ovocitos se tiñeron con 1% lacmoid en 45% (v/v) de ácido acético y examinados bajo un microscopio de contraste de fase.

Los parámetros de maduración fueron analizados a las 44 h de incubación. Los ovocitos con los cromosomes en metaphase II y el PB1, fueron considerados como ovocitos maduros.

Los parámetros de fecundación fueron evaluados a las 18 h después de la inseminación, para ello, un grupo de cigotos fueron fijados y teñidos como se ha descrito anteriormente. Los ovocitos con el citoplasma roto o con apariencia anormal fueron considerados como ovocitos degenerados. Los ovocitos fueron considerados como penetrados cuando tenían una o más de una cabeza de espermatozoide descondensada y/o pronúcleo masculino, estando presente su correspondiente flagelo y los dos cuerpos polares.

El criterio de clasificación de los pronúcleos con morfología normal se basó en el tamaño de los pronúcleos y las características de la cromatina en el interior del mismo, como por ejemplo la dispersión de la cromatina y la intensidad de tinción. Los parámetros de fecundación evaluados fueron los siguientes: penetración (número de ovocitos penetrados/total de ovocitos inseminados), monospermia (número de ovocitos que contienen sólo un prónucleo masculino/total de ovocitos penetrados), número de espermatozoides/ovocito (número medio de espermatozoides por ovocito penetrado), y
eficiencia de la fecundación (número de ovocitos monóspermicos/total de ovocitos inseminados).

Los parámetros de valoración del desarrollo y calidad embrionaria fueron evaluados en día 2 y en día 7 después de la inseminación, mediante un estereomicroscopio, evaluando los siguientes parámetros: divididos (número de 2-4 células/total cultivados) y formación de blastocistos (número de blastocistos/total cultivados), respectivamente. Sólo aquellos embriones con divisiones en 2-4 células fueron considerados como divididos, y sólo aquellos embriones con un claro blastocele fueron considerados como blastocistos.

El número total de células por blastocisto fue un indicador de la calidad embrionaria. Para el conteo de las células de los blastocistos fueron montados sobre portaobjetos para ser teñidos con 10 μg/mL H342 en medio de montaje compuesto por glicerol: DPBS (3:1). El número total de células en cada blastocisto fue determinado usando un microscopio de fluorescencia.

Activación de ovocitos madurados in vitro y cultivo in vitro

Los ovocitos maduros y denudados fueron depositados en medio de activación compuesto por 0’3 M manitol, 1’0 mM CaCl₂·H₂O, 0’1 mM MgCl₂·6H₂O, and 0’5 mM Hepes en un portaobjetos (modelo BTX #450-1), y fueron expuestos a impulsos eléctricos entre electrodos de 0,5 mm de diámetro de acero inoxidable de 1 mm de distancia. Los ovocitos fueron sometidos a 2 pulsos DC de 1’2 kV/cm durante 30 μseg usando un electromanipulador celular BTX ECM 2001 (BTX, San Diego, CA, USA). A continuación, los ovocitos activados fueron lavados 3 veces y transferidos en grupos de 30-40 ovocitos por porcillo en placas de 4 pocillos con 500 μL de medio de cultivo (NCSU-23 suplementado con 0’4% BSA) cubierto de aceite mineral. Los ovocitos fueron incubados durante 7 días con 5% CO₂ en aire, 39°C y 95-100% de humedad relativa.
Obtención de ovocitos, cigotos, 2-4 células y blastocistos in vivo

Animales

Todos los procedimientos experimentales realizados fueron previamente evaluados y aprobados por el comité bioético de la Universidad de Murcia (España), y de acuerdo con la Directiva 2010/63/EU EEC para la experimentación animal.

Para la obtención de las muestras, se emplearon cerdas híbridas de la misma línea genética (Landrace x Large White) procedentes del destete y con un número de partos comprendido entre 2 y 6. Las cerdas procedían de una granja comercial (Agropor S.L., Las Torres de Cotillas, Murcia, España), en cuyas instalaciones se mantienen alojadas en jaulas individuales en el interior de una nave con ventilación controlada. La alimentación consistía en una ración comercial proporcionada en dos tomas diarias, mientras que el agua era administrada ad libitum.

Detección del estro e inseminaciones

La detección del estro se llevó a cabo mediante la exposición de las cerdas a la presencia de un verraco adulto. Las cerdas que presentaron el reflejo de inmovilización realizado por un operario con amplia experiencia, fue considerada en celo. La detección se realizó dos veces al día a partir del segundo día posterior al destete. Las cerdas donantes fueron inseminadas artificialmente a las 12 y a las 24 h después del inicio del celo. Para ello se emplearon dosis de inseminación preparadas con semen fresco obtenido de al menos dos verracos maduros de Pietrain con fertilidad contrastada. Las dosis fueron preparadas en el Centro de Inseminación Artificial de Agropor S.L. a una concentración de $3 \times 10^9$ espermatozoides diluidos en 100 mL con BTS (Beltsville Thawing Solution; Pursel and Johson, 1975).

Medio de recogida de embriones

El medio de recogida fue TL-Hepes PVA modificado (Funahashi et al., 2000), suplementado con 10 mM Hepes.
Recogida de muestras

La recogida de muestra se realizó mediante la laparatomía de las cerdas donantes como fue descrito por Martínez y cols. (2004). En día 1 (Día 0 = inicio del celo) los folículos preovulatorios (> 8 mm de diámetro) fueron aspirados usando una aguja de 18 g conectada a una jeringa de 10 mL para obtener ovocitos maduros. En día 2 del ciclo estral, los oviductos de cada animal fueron lavados con 10 mL de medio de recogida para obtener los cigotos y embriones 2-4 células. Los blastocistos fueron recogidos en día 6 del ciclo estral mediante lavado de los cuernos uterinos con 30 mL de medio de recogida. Los ovocitos y embriones recogidos fueron visualizados bajo estereomicroscopio a 60X para evaluar el estadio de desarrollo embrionario y la calidad embrionaria. Sólo aquellos ovocitos y embriones que mostraron un aspecto morfológico bueno o excelente y un nivel de desarrollo acorde al día de la recogida fueron seleccionados para la experiencia. Posteriormente, los ovocitos y embriones seleccionados fueron lavados tres veces en medio de recogida y depositados en tubos Eppendorf de 1’5 mL con el mismo medio siendo transportados al laboratorio en la Universidad de Murcia dentro de un incubador con temperatura controlada a 39°C, en las 2 h siguientes al momento de la recogida.

Estudio de la distribución mitocondrial con microscopía confocal

Ovocitos, cigotos, 2-4 células y blastocistos in vivo e in vitro, fueron teñidos con MitoTracker Deep Red 633 (M22426; Molecular Probes, Leiden, the Netherlands) para evaluar la distribución de las mitocondrias activas. El fluorocromo fue diluido con dimetilsulfóxido a una concentración de 1 mM/L y almacenado a -20°C. Los ovocitos y embriones fueron teñidos en medio de maduración o de cultivo respectivamente, con 0.5 µM/L MitoTracker Deep Red durante 30 min a 39°C con 5% CO₂ en aire y 95-100% de humedad relativa. Tras la tinción, las muestras fueron lavadas en medio de maduración o cultivo, tres veces durante 20 min a 39°C con 5% CO₂ en aire y 95-100% de humedad relativa. A continuación, los ovocitos y embriones fueron fijados en paraformaldehído al 4% en una solución de cacodilato sódico (pH= 7.2-7.4) durante 2 h a temperatura ambiente. Las muestras fijadas fueron lavadas tres veces durante 5 min en solución salina fosfatada suplementada con 3 mg/mL de BSA. Tras el lavado final, los ovocitos y los embriones fueron montados en portaobjetos con 4 µL Vectashield (Vector, Burlingame, CA, USA) suplementado con 10 µg/mL H342 para la tinción de los núcleos. Las muestras fueron
observadas usando microscopía confocal láser (Leica TCS SP, Wetzlar, Germany). Los diferentes fluorocromos fueron excitados usando una combinación apropiada de excitación y barrera, con un láser de Argón-Criptón para la excitación de MitoTracker Deep Red (644 nm) y un láser ultravioleta para la excitación del H342 (354 nm). Las imágenes obtenidas en los diferentes cortes secuenciales se unieron y se registraron en formato digital. A continuación, dichas imágenes se visualizaron usando Adobe Photoshop 7 (Adobe Systems, San Jose, CA, USA) y se procedió a evaluar la distribución de las mitocondrias activas.

**Extracción de DNA mitocondrial y cuantificación PCR real-time**

El número de copias de DNA mitocondrial (mtDNA) fue determinado como describen Spikings y cols. (2007). Los ovocitos, cigotos, 2-4 células y blastocistos fueron individualmente colocados en eppendorf que contenía 50 µL de agua libre de nucelasa. Las muestras fueron sometidas a dos congelaciones y a sus dos consecutivas descongelaciones, para liberar el DNA. Los fragmentos del mtDNA molde constan de 296 pb (GeneBank accession number NC_012095.1) y fueron amplificados mediante la reacción en cadena la polimerasa (PCR) usando los siguientes primers (forward primer: CTC AAC CCT AGC AGA AAC CA and reverse primer: TTA GGT GGT ATC GGA ATC G). A continuación, el producto de la PCR, se resolvió en un gel de agarosa al 2% y se extrajo el DNA de la banda resultante usando QiaQuick Gel Extraction kit (Qiagen, London). Se asumió que el 21.73 ng de producto de 296 pb PCR contenía $6.79 \times 10^{10}$ de la doble cadena de mtDNA (Tamaño del producto de la PCR = 296 pb; Concentración = 21.73 ng/µL = 21.73x10^{-9} g/µL; Cálculos: $(21.73 \times 10^{-9} \text{ g/µL} / [296 \times 660]) \times 6.022 \times 10^{23} = 6.79 \times 10^{10}$ moléculas/µL). Se realizaron diluciones decimales seriadas para construir una curva estándar para la PCR cuantitativa (qPCR). La qPCR se llevó a cabo mediante Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). La muestra para la reacción de la qPCR contenía 12.5 µL 2X Power SYBR Green (Applied Biosystems, Warrington, UK), los primers en la concentración adecuada para el mtDNA (16.5 pM de cada uno), 2 µL de cada muestra o de la curva estándar y agua estéril libre de nucelasa hasta completar los 25 µL.

Las condiciones de la reacción fueron las siguientes: 1 ciclo a $95^\circ \text{C}$ por 10 min seguido de 50 ciclos de $94^\circ \text{C}$ por 10 seg, $56^\circ \text{C}$ por 15 seg, y $72^\circ \text{C}$ por 30 seg. Por cada replicado de qPCR, una curva estándar era utilizada, usando 5 diluciones decimales seriadas ($10^{2}-10^{7}$ copias). El valor de $C_t$ (Ciclo umbral o threshold cycle) de cada muestra se comparó...
con la curva estándar, lo que permite el cálculo del número de copias de mtDNA en cada muestra. La curva de disociación fue analizada para asegurar la especificidad de los productos amplificados. Todas las reacciones se realizaron por duplicado y la cantidad media de mtDNA se calculó utilizando el software 7300 ABI system sobre la base de la curva estándar.

**Estudio inmunohistoquímico de microtúbulos y cromosomas**

Los ovocitos fueron fijados en una solución al 4% de formaldehído-PBS y permeabilizados con Triton X-100 (2.5% (v/v) en PBS) durante 15 min, simultáneamente fueron teñidos para la detección α-tubulina y para la detección de cromatina (Morató y cols., 2008). La fijación y las incubaciones se realizaron a 37°C. Los ovocitos fijados fueron incubados con el anticuerpo monoclonal anti-α-tubulina (isotipo de ratón IgG1) (1:4.000) durante 90 min, seguido de una incubación con el anticuerpo secundario, Alexa Fluor 488 conjugado de cabra IgG anti-ratón (Molecular Probes-Invitrogen, UK) (1:500), durante 1 h. Entre cada incubación, se realizaron tres lavados de 5 min en PBS precalentado. Posteriormente, los ovocitos fueron montados individualmente en portaobjetos con Vectashield como medio de montaje con 1.5 μg/mL DAPI para teñir la cromatina de los ovocitos (Vector Laboratories, Inc., Burlingame, CA, USA). Cada portaobjetos fue sellado con laca de uñas y protegido de la luz hasta su observación. Los controles negativos para α-tubulina se realizaron utilizando sólo el anticuerpo secundario. Un microscopio láser confocal de barrido (Leica TCS-SP2-AOBS) fue utilizado para examinar la tubulina (Alexa Fluor 488, de excitación 488 nm) y la cromatina (DAPI; de excitación 405 nm). Las imágenes fueron registradas digitalmente. La morfología de los microtúbulos y los cromosomas se clasificaron en tres categorías: 1) Eje normal: estructura típica del huso meiótico con los cromosomas agrupados en un paquete en la placa metafásica y los microtúbulos que cruzan la longitud del eje de polo a polo. 2) Eje anormal: microtúbulos no forman típicos husos meióticos. Con cromosomas desordenados o desplazamientos de la placa metafásica. 3) Ausencia del eje: alrededor de los cromosomas no hay presencia de microtúbulos.
Visualización de husos meióticos mediante microscopio de luz polarizada

Los ovocitos fueron distribuidos en gotas de 10 μL TCM199-Hepes-FCS (TCM199-Invitrogen, Barcelona, Spain, 25 mM Hepes and 10% Fetal Calf Serum), cubiertas con aceite mineral sobre placas de Petri de cristal. La visualización del huso meiótico fue realizada usando un microscopio invertido (Olympus IX71, Japan) a 200X con un sistema Oosight (CRI, Woburn, MA, USA) que permite ver la estructura interna de los ovocitos, equipado con un filtro óptico sintonizable de cristal líquido, un polarizador circular/ filtro verde de interferencia óptica, una cámara científica grado CCD y software apropiado para la adquisición y análisis de imágenes. Durante toda la evaluación, los ovocitos se mantuvieron a 38°C en una termoplatina calentada (Tokai Hit, Japón). Cada ovocito fue rotado utilizando dos micromanipuladores (Eppendorf, Alemania) y micropipetas que ayudaron a sostener y girar los ovocitos hasta que el huso meiótico fue visualizado como una mancha blanca clasificados como “señal positiva”, o en caso contrario, clasificados como “sin señal positiva”.
DISEÑO EXPERIMENTAL Y RESULTADOS

Objetivo 1: Evaluar los efectos de la exposición a la tinción con Hoechst 33342 y a la luz ultravioleta, sobre la fertilidad y posterior capacidad de desarrollo de ovocito porcinos madurados in vitro.

Diseño experimental

Experimento 1.1: Efecto del H342 y de la luz UV durante 30 seg.

En este experimento, fueron evaluados tanto los efectos individuales como los efectos combinados de la exposición a la tinción con H342 durante 12 min y a la luz UV durante 30 seg sobre el desarrollo de ovocitos porcinos madurados in vitro. Tras la maduración, grupos de 30 ovocitos fueron incubados durante 12 min en 500 μL de medio maduración con 0 ó 5 μg/mL H342. Tras la incubación, los ovocitos fueron lavados en medio de maduración y colocados individualmente en gotas de 20 μL del mismo medio cubiertas por aceite mineral para ser expuestas o no, según el diseño experimental, a luz UV.

Un total de 1.388 ovocitos madurados in vitro procedentes de 5 replicados fueron distribuidos al azar en los siguientes grupos: Grupo 1: ovocitos sin tratar (Control), Grupo 2: ovocitos teñidos con H342, Grupo 3: ovocitos teñidos con H342 y expuestos a luz UV durante 30 seg, Grupo 4: ovocitos expuestos a luz UV durante 30 seg. Tras los tratamientos, los ovocitos de cada grupo experimental fueron inseminados y cultivados durante 18h (n = 437) para evaluar los parámetros de fecundación ó 7 días (n = 951) para determinar los parámetros de desarrollo embrionario.

Experimento 1.2: Efecto del H342 y de la luz UV durante 5 seg.

Los resultados obtenidos en el experimento 1.1 mostraron una limitación en el desarrollo embrionario de los ovocitos expuestos a la tinción con H342 y a luz UV por 30
seg, por ello este experimento fue diseñado para evaluar los efectos de la exposición corta a luz UV (5 seg) sobre los parámetros de fecundación y posterior desarrollo embrionario.

Un total de 1.835 ovocitos madurados in vitro procedentes de 6 replicados fueron distribuidos como se describió en el experimento 1.1. La diferencia fue que en los grupos con exposición a luz UV, la duración de la radiación fue sólo de 5 seg. Tras los tratamientos, los ovocitos fueron inseminados y cultivados durante 18 h (n = 749) para examinar los parámetros de fertilización y durante 7 días (n = 1.086) para evaluar los parámetros de desarrollo embrionario.

**Resultados**

**Experimento 1.1.**

El rango de maduración de los ovocitos osciló de 75'2 ± 6'7% a 82'9 ± 6'4% entre los distintos replicados, sin que existieran diferencias estadísticas significativas. La efectividad de la técnica de la fecundación in vitro usada en este estudio fue similar a la obtenida anteriormente en otros estudios en nuestro laboratorio, con más del 90% y del 50% en tasas de penetración y monospermia, respectivamente y con un porcentaje de eficiencia aproximadamente del 44%. Las tasas de penetración (P < 0'001) y la monospermia (P < 0'04) resultaron más bajas en los ovocitos expuestos a H342/UV (80'7 ± 4'5% y 30'7 ± 5'4%, respectivamente) que en los ovocitos del grupo Control (94'9 ± 4'3% y 50'0 ± 4'9%, respectivamente), por ello, la eficiencia de la fertilización resultó menor (P < 0'04). En cada grupo, todos los ovocitos fecundados formaron pronúcleos femeninos con morfología y configuración de cromatina normal. Independientemente del tratamiento, en más de un 97% de los ovocitos fecundados se desarrollaron pronúcleos masculinos con configuración normal. Además, en menos de un 8% de los ovocitos examinados, fueron observados cabezas de espermatozoides descondensadas en el interior del citoplasma, sin diferencias entre grupos. En estos ovocitos, el número de cabezas descondensadas por ovocito osciló entre 1 y 3. Los ovocitos expuestos a H342/UV obtuvieron un menor (P < 0’001) porcentaje de divididos (49'8 ± 2'9%) y de formación de blastocistos (7'7 ± 2'9%) que en los otros grupos (rango: de 73'8 ± 2'9% a 77'7 ± 2'9% y de 22'3 ± 2'9% a 30'9 ± 3'0%, respectivamente). No se observaron diferencias en el número total de células por blastocisto entre los distintos grupos.
Experimento 1.2.

No se observaron diferencias significativas en el porcentaje de maduración entre los distintos replicados (rango: de 71'6 ± 6'4% a 78'8 ± 7'2%). Independientemente del tratamiento, tanto los pronúcleos femeninos como los masculinos se desarrollaron con morfología normal en más del 96% de los ovocitos fertilizados y, adicionalmente, se observaron cabezas de espermatozoides en menos de un 6% de los ovocitos. Los porcentajes de penetración y monospermia, el número de espermatozoide por ovocito, la eficiencia final de la técnica de fecundación in vitro y la tasa de división no se vieron afectados por los diferentes tratamientos. Sin embargo, los ovocitos expuestos a H342 en combinación con la luz UV durante 5 seg mostraron menor (P < 0’02) porcentaje de formación de blastocistos (15’2 ± 4’5%) que en los otros grupos (rango: de 26’1 ± 4’5% a 30’7 ± 4’5%). No existieron diferencias significativas entre los grupos experimentales en el número total de células por blastocisto.

Estos resultados muestran que la exposición de los ovocitos a H342 en combinación con luz UV por periodos tan cortos como 5 seg tiene un efecto perjudicial sobre la capacidad de desarrollo de los ovocitos porcinos madurados in vitro.

Objetivo 2: Evaluar los efectos de la tinción con Hoechst 33342 en combinación con luz ultravioleta sobre la distribución mitocondrial y el número de copias de DNA mitocondrial de ovocitos porcinos y de embriones preimplantacionales.

Diseño experimental

En este estudio se evaluó la distribución de las mitocondrias activas y el número de copias de mtDNA en ovocitos y embriones porcinos in vitro e in vivo.

Un total de 1.426 ovocitos madurados in vitro procedente de 3 replicados fueron divididos en no expuestos (grupo Control in vitro) y en expuestos (H342/UV) a 5 µg/mL de H342 y a luz UV durante 30 seg. Posteriormente, ambos grupos fueron sometidos a una fecundación in vitro y cultivo in vitro. En cada replicado, un grupo al azar de ovocitos madurados in vitro, cigotos, 2-4 células y blastocistos fueron usados para determinar la
distribución de mitocondrias activas y la determinación del número de copias de mtDNA, y el resto se utilizó para valorar los parámetros de desarrollo embrionario. Sólo aquellos ovocitos intactos que poseían el primer corpúsculo polar extruido y los ovocitos fecundados formados por una sola célula y por dos cuerpos polares visibles, fueron seleccionados como ovocitos maduros y cigotos, respectivamente. Los ovocitos y embriones in vivo (n = 150) fueron obtenidos a partir de 10 cerdas en dos replicados (2-3 cerdas/tipo de muestra) y usados como controles. Para el estudio de la distribución mitochondrial, todas las muestras (ovocitos y embriones) fueron teñidas y fijadas hasta el momento de su evaluación en el microscopio confocal. Para la determinación del número de copias de mtDNA las muestras individualmente fueron suspendidas en 50 μL de agua estéril libre de nucleasa y congelados a -80°C hasta su análisis.

**Resultados**

_Efecto de la exposición a H342 y luz UV sobre el desarrollo de ovocitos fecundados in vitro._

Un total de 1.075 ovocitos madurados in vitro, fueron inseminados y cultivados durante 7 días para evaluar su desarrollo embrionario. Los ovocitos expuestos a H342/UV mostraron un menor (P < 0.001) porcentaje de división (40.9 ± 12.2%) y de formación de blastocistos (10.6 ± 1.6%) que los ovocitos derivados del grupo Control (62.4 ± 12.2% y 24.4 ± 1.6%, respectivamente). No se observaron diferencias en el número total de células por blastocisto entre grupos.

_Efectos de la exposición a H342 y luz UV sobre la distribución de mitocondrias activas en ovocitos maduros y embriones preimplantacionales._

En la mayoría de los ovocitos madurados in vivo (11/13; 84.6%), las mitocondrias activas estaban distribuidas por todo el citoplasma, aunque eran más abundantes en la zona pericitoplasmática. Además, los cuerpos polares mostraron una fuerte tinción. Esta típica distribución se observó en un 90.9% de los ovocitos madurados in vitro y en un 68.4% de los ovocitos expuestos a H342/UV (20/22 y 13/19, respectivamente). Aquellos ovocitos que presentaron pobre tinción o no presentaron tinción fueron considerados como ovocitos maduros con distribución anormal de mitocondrias. Después de la fecundación, el 100% de los cigotos in vivo (18/18) mostraron agregados de mitocondrias asociados a los
pronúcleos y además cerca de la membrana plasmática. También se observó una fuerte tinción mitocondrial en el primer y en el segundo cuerpo polar. Este patrón de distribución fue observado en el 75% (6/8) de los cigotos monospérmicos obtenidos in vitro y 58'3% (7/12) en los cigotos monospérmicos expuestos a H342/UV. Los cigotos in vitro sin mitocondrias alrededor de los pronúcleos fueron considerados como cigotos con distribución mitocondrial anormal. Durante las primeras divisiones, en la mayoría de los embriones divididos en 2-4 células in vivo (25/26; 96'2%) e in vitro Control (24/27; 88'9%), las mitocondrias se distribuyeron homogéneamente por todo el citoplasma, remarcando la periferia de cada blastómero, y formando agregados en la región nuclear. En contraste con esta distribución, sólo en 13/30 (43'3%) de los embriones en 2-4 células derivados de ovocitos expuestos a H342/UV presentaron este patrón de distribución mitocondrial. Además, en un 20% (6/30) de los embriones del grupo expuesto a H342/UV se hallaron fragmentos anucleados con fuerte tinción para mitocondrias activas y, además, en un 36'6% (11/30) presentaban blastómeros con pobre o escasa tinción para las mitocondrias. En los blastocistos, de todos los grupos experimentales, se observaron mitocondrias activas distribuidas homogéneamente tanto en la masa celular interna como en el trofoectodermo.

**Efectos del H342 y de la luz UV sobre el número de copias de mtDNA en ovocitos maduros y embriones preimplantacionales.**

No se observaron diferencias significativas entre el número de copias de mtDNA de ovocitos maduros in vivo, in vitro Control e in vitro expuestos a H342/UV (rango: de 347.023 ± 46.334 a 400.615 ± 89.487). Sin embargo, se obtuvo un menor (P < 0'04) número de copias de mtDNA en los cigotos (85.909 ± 19.503) y en 2-4 células (110.280 ± 23.082) y un mayor (P < 0'02) número de copias de mtDNA en los blastocistos (557.115 ± 176.717) obtenidos a partir de ovocitos expuestos a H342/UV que en embriones obtenidos in vivo (162.324 ± 25.508; 180.878 ± 17.009; y 167.287 ± 33.379, respectivamente). No se observaron diferencias entre los ovocitos y embriones obtenidos in vivo e in vitro.

Cuando comparamos el número de copias de mtDNA obtenido dentro de cada grupo experimental (in vivo o in vitro, expuesto o no expuesto a H342/UV), las diferencias significativas se observaron en función a su etapa de desarrollo. En el grupo experimental in vivo, la media de las copias de mtDNA (400.615 ± 89.487) obtenida en los ovocitos maduros fue significativamente mayor (P < 0'02) que en cigotos, 2-4 células y blastocistos (162.324 ±
25,508; 180.878 ± 17.009; y 167.287 ± 33.379, respectivamente). En el grupo experimental in vitro, la media de las copias de mtDNA fueron 347.023 ± 46.334 y 370.441 ± 77.126 para el Control y para aquellos ovocitos que fueron expuestos a H342/UV, respectivamente; este valor decreció (P < 0.02) en cigotos (100.575 ± 20.895 y 85.909 ± 19.503, respectivamente) y en 2-4 células (176.192 ± 26.607 y 110.280 ± 23.082, respectivamente). Sin embargo, las copias de mtDNA en blastocistos aumentaron alcanzando valores de 320.056 ± 52.590 y 557.115 ± 176.717 para el Control y H343/UV, respectivamente, cuyas medias no difirieron de las obtenidas para los ovocitos maduros.

Estos resultados muestran que los ovocitos porcinos expuestos simultáneamente a H342 y luz UV presentan una reducción en su capacidad de desarrollo, un patrón anormal de distribución mitocondrial y una disminución en el número de copias de mtDNA de los embriones divididos obtenidos. Por ello, debería ser replanteado el uso de H342 y de luz UV como método de ayuda y/o confirmación durante la enucleación en la transferencia nuclear.

**Objetivo 3: Investigar la efectividad de un fluorocromo de longitud de onda larga (SYBR-14) para visualizar los cromosomas maternos de ovocitos porcinos madurados in vitro y los efectos de esta tinción en combinación con la excitación de la fluorescencia sobre la fecundación in vitro y posterior desarrollo embrionario de los ovocitos.**

**Diseño experimental**

**Experimento 3.1: Visualización de la placa metafásica y de PB1 de ovocitos madurados in vitro expuestos a diferentes concentraciones SYBR-14 y tiempos de incubación.**

Este experimento fue diseñado con el fin de determinar las condiciones óptimas para enucelar los ovocitos utilizando SYBR-14, para ello se evaluó el efecto de diferentes concentraciones de SYBR-14 (Invitrogen, Eugene, OR, USA) y de distintos tiempos de incubación sobre la visualización de los cromosomas en metaphase II (MII) y PB1 de ovocitos maduros. Inmediatamente después de la maduración, un total de 913 ovocitos denudados procedentes de 3 replicados fueron incubados en 100 μL de medio de maduración con distintas concentraciones (1, 3, 5 y 7 μg/mL) de SYBR-14 y tiempos de incubación (5, 10 y 30 min) en un diseño experimental 4x3. A continuación, los ovocitos
fueron lavados en medio de maduración y distribuidos individualmente en gotas de 20 μL del mismo medio cubiertas de aceite mineral precalentado para ser expuesto a la fluorescencia según el diseño experimental. La placa metafásica y PB1 fueron visualizadas por excitación con un microscopio de fluorescencia (Nikon Eclipse TE2000-S®). La fluorescencia se observó usando un filtro de excitación de 485 nm y un filtro de emisión de 520 nm. Los ovocitos fueron considerados correctamente teñidos cuando se pudo observer la placa metafásica y PB1. Para confirmar los resultados de la visualización, los ovocitos fueron fijados en ácido acético/etanol, teñidos con lacmoid y examinados con un microscopio de contraste de fases como se ha descrito anteriormente. La eficiencia de visualización fue definida como el número de ovocitos con placa metafásica y PB1 visualizados mediante fluorescencia dividido por el número de ovocitos con placa metafásica y PB1 visualizados mediante fijación y tinción. La concentración y el tiempo de incubación óptimos obtenidos en este experimento, fue utilizado para comprobar la eficiencia de la enucleación. Tras 44 h de maduración, 86 ovocitos denudados fueron incubados durante 10 min en medio de maduración con 5 μg/mL SYBR-14. Tras la tinción, los ovocitos fueron alojados en gotas individuales de 20 μL del mismo medio cubiertas de aceite mineral precalentado. Cada ovocito fue rotado con ayuda de dos micromanipuladores (Eppendorf, Germany). Los cromosomas en MII y los PB1 fueron aspirados usando una pipeta de cristal biselada de 17 μm. Para confirmar la adecuada enucleación, todos los ovocitos enucleados fueron montados sobre portaobjetos en 4 μL de glicerol-DPBS (3:1) con 10 μg/mL H342, y evaluados usando un microscopio de fluorescencia. La eficiencia de enucleación fue definida como el número de ovocitos sin DNA residual después de la enucleación, dividido por el número total de ovocitos enucleados.

**Experimento 3.2: Efecto del SYBR-14 y de la exposición a la fluorescencia sobre el desarrollo competente de ovocitos madurados in vitro.**

Este estudio fue diseñado para evaluar los efectos de la tinción con SYBR-14 y su posterior excitación con fluorescencia durante 5 y 30 seg sobre el desarrollo de los ovocitos madurados in vitro. Las mejores condiciones de tinción, determinadas en el primer experimento, fueron 5 μg/mL SYBR-14 durante 10 min. Inmediatamente después de la maduración, grupos de 30 ovocitos fueron incubados durante 10 min en medio de maduración en gotas de 100 μL con 0 ó 5 μg/mL SYBR-14. Tras la tinción, los ovocitos fueron
lavados en medio de maduración y alojados en gotas individuales de 20 μL del mismo medio cubiertas de aceite mineral para ser expuesto o no a la fluorescencia, según el diseño experimental.

Un total de 1.708 ovocitos madurados in vitro fueron distribuidos al azar en los siguientes grupos experimentales: Grupo 1: ovocitos no tratados (Control), Grupo 2: ovocitos expuestos a la tinción con SYBR-14, Grupo 3: ovocitos expuestos a la tinción con SYBR-14 y posterior fluorescencia durante 5 seg y Grupo 4: ovocitos expuestos a tinción con SYBR-14 y posterior fluorescencia durante 30 seg. Después de los tratamientos, los ovocitos de cada grupo experimental fueron inseminados y cultivados, durante 18 h (n=510) para evaluar los parámetros de fecundación y durante 7 días (n=1.198) para determinar los parámetros de desarrollo embrionario.

En un estudio complementario se evaluaron los parámetros de fecundación y de desarrollo embrionario de ovocitos expuestos (5 seg ó 30 seg) y no expuestos exclusivamente a la fluorescencia. Para dicho estudio se realizó el mismo diseño experimental descrito anteriormente a excepción de la incubación con SYBR-14. Un total de 782 ovocitos madurados in vitro procedentes de tres replicados fueron divididos al azar en 3 grupos: Grupo 1: ovocitos sin tratar (Control), Grupo 2: ovocitos expuestos a fluorescencia durante 5 seg y Grupo 3: ovocitos expuestos a fluorescencia durante 30 seg. Después de los tratamientos, los ovocitos fueron inseminados y cultivados durante 12 h (n = 280) para examinar los parámetros de fertilización y durante 7 días (n = 502) para examinar los parámetros de desarrollo embrionario.

**Resultados**

**Experimento 3.1.**

El análisis de la varianza mostró que la concentración del fluorocromo, el tiempo de incubación y la interacción entre ambos factores influyeron en los porcentajes de visualización de placa metafásica y PB1. Los mayores porcentajes de eficiencia de visualización fueron obtenidos en ovocitos teñidos con 5 μg/mL SYBR-14 durante 10 min y 7 μg/mL SYBR-15 durante 5 min. No se observó aumento de la intensidad de la fluorescencia con tiempos de incubación mayores de 10 min ni con concentraciones mayores de SYBR-14.
Las mínimas condiciones de tinción requeridas para la visualización de placa metafásica y PB1 escogida durante este experimento fue 5 μg/mL de SYBR-14 durante 10 min, con lo que se obtuvo una eficiencia de visualización del 81'3 ± 4'9%. Además, la emisión de la fluorescencia permaneció observable durante 40 min, y 82 de 86 ovocitos fueron adecuadamente enucleados con una eficiencia de enucleación del 95'3%.

**Experimento 3.2.**

La media de los porcentajes de maduración después de 44 h de incubación fue 80'7 ± 6'1%; sin diferencias significativas entre los replicados. Tras 18 h del tratamiento, en los grupos experimentales que fueron expuestos al SYBR-14 y a la fluorescencia se observó mayor (P < 0'002) porcentaje de ovocitos degenerados que en el resto de grupos experimentales. Para evaluar los efectos específicos de SYBR-14 y la luz de fluorescencia sobre los parámetros de fecundación, los ovocitos degenerados e inmaduros fueron excluidos del análisis, y por lo tanto sólo se tuvieron en cuenta los ovocitos maduros. Los porcentajes de penetración (rango: de 66'7 ± 6'1% a 76'9 ± 3'6%), monospermia (rango: de 55'7 ± 4'9% a 58'9 ± 6'6%), el número de espermatozoide por ovocito penetrado (rango: de 1'6 ± 0'1 a 1'8 ± 0'1) y el porcentaje de eficiencia de la fecundación (rango: de 38'3 ± 6'3% a 43'9 ± 4'2%) no se vieron influenciados por los diferentes tratamientos. La exposición única a SYBR-14 no produjo efecto adverso sobre el desarrollo in vitro de los ovocitos.

Los índices de división y de formación de blastocistos fueron menores (P < 0'001) para los ovocitos expuestos a SYBR-14 y a fluorescencia durante 5 seg (31'5 ± 2'7% and 7'4 ± 1'5%, respectivamente) y durante 30 seg (14'9 ± 2'1% and 2'0 ± 0'1%, respectivamente) que los ovocitos pertenecientes al grupo Control (66'2 ± 2'7% and 27'8 ± 2'6%, respectivamente). La combinación de SYBR-14 y la exposición a fluorescencia durante 5 seg y 30 seg produjo un descenso (P < 0'02) en la tasa de embriones divididos que llegaron a alcanzar el estadio de blastocistos de 18'6 puntos y 30'6 puntos, respectivamente, comparados con el Control.

Los resultados obtenidos en el experimento adicional mostraron que la exposición de ovocitos maduros exclusivamente a la fluorescencia no afectó a las tasas de degeneración (rango: 0'0 – 2'3%), fertilización ni al desarrollo embrionario.
Nuestros resultados demuestran que la combinación de la tinción con SYBR-14 y la exposición a la fluorescencia por periodos tan cortos como 5 seg ejerce claros efectos perjudiciales sobre la viabilidad y la capacidad de desarrollo de ovocitos porcinos.

**Objetivo 4:** Evaluar la eficiencia del Microscopio de Luz Polarizada para detectar y asistir la retirada del huso meiótico de ovocitos porcinos madurados *in vitro*, y examinar los efectos del microscopio de luz polarizada sobre el desarrollo competente del ovocito.

**Diseño experimental**

*Experimento 4.1:* Evaluación de la eficiencia de PLM para detectar proteína de los microtúbulos polimerizados en ovocitos porcinos madurados *in vitro*.

Los ovocitos fueron valorados con un microscopio de luz polarizada para detectar proteína de los microtúbulos polimerizados de los ovocitos, las cuales podrían formar parte del huso meiótico. Un total de 117 ovocitos procedentes de 7 replicados fueron analizados. La presencia de la proteína y del huso meiótico fue confirmada individualmente en cada ovocito mediante técnicas de inmunotinción y detección de cromatina. La señal positiva detectada con PLM de los ovocitos fue evaluada por el software Oosight para determinar un valor predictivo de la densidad y conformación del microtúbulo.

*Experimento 4.2:* Examinar el efecto de PLM sobre el desarrollo competente de los ovocitos porcinos madurados *in vitro*.

Un total de 160 ovocitos en MII, de un total de 4 replicados, fueron distribuidos en grupos de 10 ovocitos por gota de 10 µL para ser posteriormente expuestos o no expuestos a PLM durante 10 min. A continuación, los ovocitos fueron activados partenogenéticamente *in vitro*. 
Experimento 4.3: Selección de ovocitos mediante PLM y evaluación del efecto de dicha selección sobre la eficiencia de la fertilización y desarrollo embrionario.

Los ovocitos fueron expuestos a PLM evaluando la presencia o no del huso meiótico. Los ovocitos del grupo Control no fueron expuestos a PLM, por lo tanto, no fue evaluado la presencia o no del huso meiótico. Se obtuvieron 3 grupos experimentales, los ovocitos con señal positiva (PLM positivo), ovocitos con señal negativa (PLM negativo) y el grupo Control. Posteriormente, los ovocitos fueron fecundados y cultivados in vitro.

Experimento 4.4: Evaluación de la eficiencia de enucleación mediante PLM.

Los ovocitos en MII fueron teñidos con 1μg/mL H342 y distribuidos individualmente en microgotas de 20 μL. Con la ayuda de dos micromanipuladores los ovocitos fueron rotados hasta que la placa metafásica alcanzaba la posición adecuada para poder ser aspirada bajo la luz polarizada. La correcta enucleación de la placa metafásica fue comprobada y confirmada por exposición de todo el citoplasma extraído tanto con sistema de Oosight-PLM como con luz ultravioleta. Se realizaron 3 replicados del experimento.

Resultados

Experimento 4.1.

La señal PLM positiva fue detectada en 98’2 % de los ovocitos, y en 96’5% de ellos se encontraban en MII. Todos los ovocitos en MII, menos uno, fueron positivos a PLM. Hubo una correlación positiva ($r = 1; P < 0'0001$) entre la señal obtenida por PLM y la presencia de proteína de microtúbulos polimerizados como se confirmó con la inmunotinción. A través de la inmunotinción se observó que en el 92’9% de los ovocitos en MII presentan la estructura típica del huso meiótico (barrelshape spindle), y sólo en un 6’2% de los ovocitos en MII presentaban una conformación anormal del huso meiótico.

Los valores de retardo obtenidos en los ovocitos porcinos madurados in vitro después de ser expuestos al PLM oscilaron en un rango de 1’87 a 6’38 nm, además la media de los valores de retardo obtenidos para los ovocitos con huso meiótico normal (barrel
shape spindle) (3'9 ± 0'1 nm) no fue diferente de la media obtenida en los ovocitos con huso meiótico anormal (3'2 ± 0'4 nm), evaluado mediante inmunotinción.

Experimento 4.2.

Tras ser examinados por PLM, los ovocitos fueron activados partenogenéticamente y posteriormente cultivados in vitro. No se observaron diferencias significativas entre los ovocitos expuesto al PLM y el Control en los porcentajes de división (83'7 ± 1'5% y 84'4 ± 1'5%, respectivamente), número total de blastocistos (36'9 ± 3'6% y 41'2 ± 3'6%, respectivamente) y número de blastocistos expandidos en día 7 (21'9 ± 1'7% y 26'2 ± 1'7%, respectivamente). Tampoco se encontraron diferencias en el número total de células por blastocisto expandido.

Experimento 4.3.

A pesar de las diferencias de resultados entre machos en fecundación in vitro, en este estudio se utilizó el semen congelado de un verraco anteriormente testado. El objetivo fue obtener embriones con capacidad de producir camadas. No se encontraron diferencias entre los distintos grupos experimentales cuando fueron valorados los porcentajes de penetración (rango: de 66'7 ± 4'8% a 72'2 ± 4'8%), monospermia (rango: de 60'6 ± 6'2% a 66'4 ± 6'2%) y eficiencia (rango: de 36'5 ± 3'8% a 42'4 ± 3'8%).

Sin embargo, el porcentaje de ovocitos que alcanzaron el estadio de MII dentro de los ovocitos del grupo PLM (80'2 ± 1'6%) fue significativamente menor que el grupo Control y el grupo PLM positivo (96'0 ± 1'6% y 100'0 ± 1'6%, respectivamente). No se encontraron diferencias significativas en los porcentajes de división entre los grupos (rango: de 53'1 ± 7'6% a 70'5 ± 5'7%). Sin embargo, con respecto a los porcentajes de blastocistos, se observaron diferencias significativas entre el grupo de PLM negativo (11'5 ± 2'4%) y el resto de grupos experimentales (Control, 25'5 ± 1'8%; PLM positivo, 27'7 ± 1'8%). No se encontraron diferencias en el número total de células por blastocisto entre el Control, PLM positivo y PLM negativo.
Experimento 4.4.

Un total de 54 ovocitos en MII fueron enucleados con el sistema Oosight con una eficiencia del 92'6%. El procedimiento fue simple y la cantidad de citoplasma eliminado en cada enucleación fue mínimo.

Estos resultados muestran que el PLM es un sistema eficiente para detectar la proteína de microtúbulos polimerizados en ovocitos porcinos madurados in vitro. Además, es una técnica eficiente para remover el huso meiótico sin ejercer ningún efecto perjudicial sobre la capacidad de desarrollo de ovocitos porcinos y puede ser usado como una herramienta para mejorar algunas tecnologías reproductivas como la transferencia nuclear en esta especie.
1. La exposición de ovocitos porcinos a Hoechst 33342 o a luz ultravioleta separadamente, no interfirió en su fertilización ni en su desarrollo embrionario. Sin embargo, la exposición combinada de Hoechst 33342 y luz ultravioleta produjo efectos perjudiciales sobre el desarrollo de los ovocitos fertilizados in vitro, siendo estos efectos más drásticos al aumentar los tiempos de exposición a la luz ultravioleta.

2. La exposición de ovocitos porcinos a Hoechst 33342 y luz ultravioleta está asociado con un patrón anormal de distribución mitocondrial y a una disminución en el número de copias de DNA mitocondrial de los embriones divididos obtenidos. Por ello, debería ser replanteado el uso de Hoechst 33342 y de luz ultravioleta como método de ayuda y/o confirmación durante la enucleación en la transferencia nuclear.

3. La combinación de la tinción con SYBR-14 y la exposición a la fluorescencia por periodos tan cortos como 5 seg ejerce claros efectos perjudiciales sobre la viabilidad y la capacidad de desarrollo de los ovocitos porcinos. Estos resultados indican que la tinción con SYBR-14 debe evitarse para enuclear en la especie porcina.

4. El Microscopio Luz Polarizada es un sistema eficiente para detectar la proteína de microtúbulos polimerizados en los ovocitos porcinos madurados in vitro sin efecto perjudicial sobre su capacidad de desarrollo. Esta técnica podría ser un método eficiente para enuclear ovocitos porcinos, susceptible de ser usado dentro del proceso de la transferencia nuclear en la especie porcina.
ABBREVIATIONS
ABBREVIATIONS

BSA: Bovine serum albumin

COCs: Cumulus oocytes complexes

DPBS: Dulbecco’s phosphate-buffered saline medium

FF: Follicular fluid

H342: Hoechst 33342

IVF: In vitro fertilization

mDPBS: Modified Dulbecco’s phosphate-buffered saline medium

MII: Metaphase II

mTBM: Modified Tris-buffered medium

mtDNA: Mitochondrial DNA

NCSU23: North Caroline State University medium

PB1: First polar body

PCR: Polymerase chain reaction

PLM: Polarized light microscopy

qPCR: Quantitative real-time PCR

ROS: Reactive oxygen species

SCNT: Somatic cell nuclear transfer

TBM: Tris-buffered medium

TL-HEPES PVA: Tyrode’s lactate – Hepes PVA

UV: Ultraviolet
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ARTICLES
ARTICLE 1

Effects of Hoechst 33342 staining and ultraviolet irradiation on the developmental competence of in vitro-matured porcine oocytes.
Effects of Hoechst 33342 staining and ultraviolet irradiation on the developmental competence of in vitro-matured porcine oocytes


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Abstract

Hoechst 33342 (H342) in combination with ultraviolet (UV) irradiation is frequently used to assist the enucleation of porcine oocytes in somatic cell nuclear transfer programs. This work evaluated the effects of H342 (5 μg/mL for 12 min) staining and/or exposure to UV irradiation on fertilisability and developmental capacity of porcine oocytes matured in vitro. In Experiment 1, a total of 1388 mature oocytes were distributed in the following groups: Group 1: oocytes without treatment (Control), Group 2: oocytes stained with H342, Group 3: oocytes stained with H342 and UV irradiated for 30 sec, and Group 4: oocytes UV irradiated for 30 sec. Oocytes from each group were exposed to thawed spermatozoa and cultured for 18 h to assess fertilization parameters or for 7 d to evaluate embryo development. Sperm penetration (P < 0.001) and monospermy (P < 0.04) were lower in oocytes exposed to H342/UV (80.7 ± 4.5% and 30.7 ± 5.4%, respectively) than in oocytes from the control group (94.9 ± 4.3 and 50.0 ± 4.9, respectively). The oocytes exposed to H342/UV showed lower (P < 0.001) cleavage (49.8 ± 2.9%) and blastocyst (7.7 ± 2.9%) rates than oocytes from the other groups (range: 73.8 ± 2.9% to 77.7 ± 2.9% and 22.3 ± 2.9% to 30.9 ± 3.0%, respectively). Experiment 2 was designed to evaluate the effect of shorter UV irradiation (5 sec). A total of 1835 mature oocytes were separated into the same groups as those of Experiment 1. The fertilization parameters and the cleavage rates were not influenced by the different treatments. However, the oocytes exposed to H342 and UV irradiation for 5 sec showed a lower (P < 0.02) rate of blastocyst formation (15.2 ± 4.5%) than the oocytes from other groups (range: 26.1 ± 4.5% to 30.7 ± 4.5%). In conclusion, our results demonstrate that the combination of H342 staining with UV irradiation has a clear deleterious effect on the developmental ability of oocytes, with the effects being more intense with increased exposure to UV irradiation.

1. Introduction

During the last decade, SCNT has been widely used to clone several mammalian species, including cattle, mice, goats, pigs, cats, rabbits, horses, rats, dogs, and ferrets [reviewed in 1,2]. Although the successful production of cloned pigs derived from somatic cells was first published in 2000 [3–5], and the number of cloned pigs increases each year, the cloning efficiency is still very low. The production of cloned pigs by SCNT includes several steps, each of which may affect the overall efficiency of the technique. One key step of SCNT is the enucleation of the recipient oocyte. Although several approaches have been described for enucleation of porcine oocytes, such as “blind” enucleation, chemical enucleation and Pol-scoper microscopy enucleation, a procedure implicating the use of bisbenzimide Hoechst 33342 (H342) staining and ultraviolet (UV) irradiation is frequently used to aid or confirm the

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enucleation procedure [3,5–12]. The UV-excitatable fluorochrome H342 (excitation 350 nm) is a cell membrane permeant and live-cell stain. This dye has been used to label oocyte chromatin because H342 strongly binds to the minor groove of DNA [13].

Although live piglets have been born from oocytes enucleated in the presence of H342 and UV light, it is widely assumed that the exposure of the recipient oocytes to H342 and UV irradiation might have detrimental effects on their DNA and cytoplasmic organelles [14]. However, the exact toxicity of H342 and UV irradiation on the oocyte is not yet fully known because only a limited number of studies have been conducted in a few species, including cattle, goat, rabbits, and mice [15–19]. These studies showed variable results depending mainly on the species and the length of irradiation. Despite the extensive use of H342 and UV light to enucleate porcine oocytes, to the best of our knowledge, there have been no studies performed in this species. The aim of this work was to evaluate the effects of H342 staining and/or exposure to UV light on fertilisability and developmental capacity of porcine oocytes matured in vitro.

2. Materials and methods

2.1. Culture media

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

The medium used for the collection of cumulus-oocyte complexes (COCs) and for washing was Dulbecco’s phosphate-buffered saline (DPBS) medium composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4 and 1.46 mM CaCl2·2H2O 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) [20] supplemented with 10% (v/v) porcine follicular fluid, 0.8 mM cysteine, 10 ng/mL EGF and 5 nM 9-cis retinoic acid. The basic medium used for fertilization was essentially the same as that used by Abeydeera and Day (1997) [21]. This medium, designated as a modified Tris-buffered medium, consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2·2H2O, 20 mM Tris (crystallized free base), 11 mM glucose and 5 mM sodium pyruvate supplemented with 0.5 mM caffeine and 0.2% BSA. The embryo culture medium was a sequential medium based on NCSU-23 supplemented with 0.4% BSA.

2.2. Recovery and maturation of cumulus-oocyte complexes

Ovaries were obtained from prepuberal gilts at a local slaughterhouse and were transported to the laboratory at 35 °C within 1 h after collection in 0.9% NaCl containing 70 μg/mL kanamycin. The COCs were aspirated from medium-sized follicles (3 to 6 mm in diameter) using an 18 ga 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 70 to 80 oocytes were transferred into each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 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 to 22 h. The oocytes were then incubated for another 20 to 22 h in maturation medium without hormones. Oocyte maturation was carried out under mineral oil at 39 °C in a humidified atmosphere of 5% CO2 in air. After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium by vortexing for 2 min at 1660 rounds/min and were washed twice in maturation medium.

2.3. In vitro fertilization

After treatments, oocytes were washed three times in pre-equilibrated fertilization medium and fertilized as described by Gil et al (2003) [22]. Briefly, groups of 30 denuded oocytes were 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 39 °C in an atmosphere of 5% CO2 in air for approximately 30 min until the addition of spermatozoa. Semen from a mature Pietrain boar was processed and cryopreserved in 0.5 mL straws, as described by Carvajal et al (2004) [23]. For each replicate, one pool of semen was made from two straws thawed in a circulating water-bath at 37 °C for 20 sec. Then, 100 μL of thawed semen was washed three times by centrifugation at 1900 X g for 3 min in mDPBS. The resulting pellet was resuspended in fertilization medium, and after the appropriate dilution, 50 μL of this sperm suspension was added to a 50 μL drop of fertilization medium containing the oocytes. The spermatozoon:oocyte ratio was 1000:1. The gametes were co-incubated at 39 °C in a humidified atmosphere of 5% CO2 in air for approximately 5 h.
2.4. In vitro culture

Presumptive zygotes were removed from the fertilization medium and washed three times in pre-equilibrated embryo culture medium. The zygotes were then transferred to a 4-well multidish (30 zygotes per well), with each well containing 500 μL of the same medium under mineral oil, and were cultured at 39 °C in a humidified atmosphere of 5% CO₂ in air for 18 h to assess the fertilization parameters or for 7 d to assess embryo development. Presumptive zygotes were cultured for the first 2 d in glucose-free NCSU-23 supplemented with 0.33 mM pyruvate and 4.5 mM lactate and then in fresh NCSU-23 medium containing 5.5 mM glucose until Day 7 (Day 0 = day of fertilization).

2.5. Assessment of maturation, sperm penetration and embryo development

To evaluate maturation and fertilization parameters, the oocytes and presumptive zygotes were mounted on slides, fixed in a solution of acetic acid:ethanol (1:3) for 48 to 72 h at room temperature, stained with 1% laccoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of X 400. The maturation rate was assessed at 44 h of incubation. The oocytes with chromosomes at metaphase-II and an extruded polar body were considered mature. Fertilization parameters were evaluated 18 h after insemination. The 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 criteria for pronuclei with normal morphology and chromatid configuration were based on pronuclei size and characteristics of the chromatid component inside the pronuclei (chromatin dispersion and staining intensity). The fertilization parameters evaluated were penetration rate (number of oocytes penetrated/total inseminated), monospermy (number of oocytes containing only one male pronucleus/total penetrated), number of spermatozoa/oocyte (mean number of spermatozoa in penetrated oocytes), and efficiency of fertilization (number of monospermic oocytes/total inseminated).

At 2 and 7 d after insemination, the cleavage rate (percentage of the number of oocytes divided to 2 to 4 cells/total cultivated) and blastocyst formation (percentage of the number of blastocysts/total cultivated), respectively, were evaluated under a stereomicroscope. An embryo that 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 H342, followed by examination using fluorescence microscopy. The total number of nuclei, which were stained with H342 and displayed blue fluorescence, was counted.

2.6. Experimental design

2.6.1. Experiment 1: Effect of H342 and ultraviolet irradiation for 30 sec

In this experiment, the individual and/or combined effects of H342 for 12 min and UV irradiation for 30 sec on the developmental ability of in vitro-matured oocytes were evaluated. Immediately after maturation, groups of 30 oocytes were incubated for 12 min in 500 μL of maturation medium containing 0 or 5 μg/mL H342. Subsequently, the oocytes were washed in maturation medium and individually allocated into drops of 20 μL of maturation medium overlaid with warm mineral oil to be exposed or not exposed to UV irradiation, according to the experimental design.

A total of 1388 in vitro-matured oocytes from five replicates were randomly allocated in the following groups: Group 1: oocytes without treatment (Control), Group 2: oocytes stained with H342, Group 3: oocytes stained with H342 and UV irradiated for 30 sec, and Group 4: oocytes UV irradiated for 30 sec.

After the treatments, the oocytes from each group were pooled, exposed to thawed spermatozoa and cultured for 18 h (n = 437) to assess fertilization parameters or for 7 d (n = 951) to evaluate embryo development.

2.6.2. Experiment 2: Effect of H342 and ultraviolet irradiation for 5 sec

Because the results from experiment 1 revealed that the exposure of H342-stained oocytes to UV irradiation for 30 sec limited their developmental competence, this study was designed to evaluate the effect of a shorter UV irradiation (5 sec) on fertilization parameters and on embryo development.

A total of 1835 in vitro-matured oocytes from six replicates were separated into the same groups as Experiment 1. The only difference was that in the groups with UV irradiation, the oocytes were subjected to radiation for only 5 sec. After the treatments, the oocytes were incubated with thawed spermatozoa and cultured for 18 h (n = 749) to assess fertilization parameters or for 7 d (n = 1086) to evaluate embryo development.
Table 1
Effect of Hoechst 33342 (H342) staining and ultraviolet (UV) irradiation for 30 sec on the fertilization parameters of mature oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. oocytes</th>
<th>Penetration %</th>
<th>Monospermy %</th>
<th>EO</th>
<th>Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>H342 UV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– –</td>
<td>124</td>
<td>94.9 ± 4.3a</td>
<td>50.0 ± 4.9a</td>
<td>1.7 ± 0.1</td>
<td>44.4 ± 4.7a</td>
</tr>
<tr>
<td>+ –</td>
<td>100</td>
<td>97.1 ± 4.3a</td>
<td>40.1 ± 5.1ab</td>
<td>1.8 ± 0.1</td>
<td>38.1 ± 4.6ab</td>
</tr>
<tr>
<td>+ +</td>
<td>100</td>
<td>80.7 ± 4.5b</td>
<td>30.7 ± 5.4b</td>
<td>1.9 ± 0.1</td>
<td>27.0 ± 5.2b</td>
</tr>
<tr>
<td>– +</td>
<td>113</td>
<td>88.6 ± 4.2ab</td>
<td>49.4 ± 4.9ab</td>
<td>1.8 ± 0.1</td>
<td>41.3 ± 4.7ab</td>
</tr>
</tbody>
</table>

Different letters within the same column represent a significant difference (at least P < 0.04). Penetration: number of oocytes penetrated/total inseminated; monospermy: number of oocytes containing only one sperm head or one male pronucleus/total penetrated; EO: number of spermatozoa/oocyte penetrated; efficiency: number of monospermic oocytes/total inseminated.

2.7. Statistical analysis

All data processing and statistical analyses were performed with SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data from all replicates were assessed by ANOVA using the MIXED procedure, according to a statistical model including the fixed effect of the treatments and the random effect of the replicates in each experiment. Maturation, penetration and monospermic rates, efficiency data, cleavage rates and blastocyst formation were modeled according to the binomial model of parameters, as described by Fisz (1980) [24], 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.

3. Results

3.1. Experiment 1: Effect of H342 and ultraviolet irradiation for 30 sec

The total oocyte maturation rate ranged from 75.2 ± 6.7% to 82.9 ± 6.4% among the replicates; the differences were not statistically significant. The effectiveness of the IVF procedure used in this study was similar to that obtained in other experiments in our laboratory, with more than 90% and 50% penetration and monospermy rates, respectively, and a final efficiency of approximately 44%. Sperm penetration (P < 0.001) and monospermy (P < 0.04) were lower in oocytes exposed to H342/UV (80.7 ± 4.5% and 30.7 ± 5.4%, respectively) than in oocytes from the control group (Table 1), and consequently, the efficiency of fertilization was also lower (P < 0.04). In each group, all oocytes fertilized formed a female pronucleus with a normal morphology and chromatin configuration. Normal male pronuclei were formed in more than 97% of the oocytes fertilized, irrespective of the treatment used. In addition to male pronuclei, decondensed sperm heads within the cytoplasm were presented in less than 8% of the oocytes, with no differences among the groups. In these oocytes, the number of decondensed sperm heads per oocyte ranged from 1 to 3. Cleavage rates and blastocyst formation are shown in Fig. 1. The oocytes exposed to H342/UV showed a lower (P < 0.001) cleavage (49.8 ± 2.9%) and blastocyst (7.7 ± 2.9%) rate than oocytes from the other groups (range: 73.8 ± 2.9% to 77.7 ± 2.9% and 22.3 ± 2.9% to 30.9 ± 3.0%, respectively). No differences were observed in the blastocyst total cell number among the groups (Fig. 1).

3.2. Experiment 2: Effect of H342 and ultraviolet irradiation for 5 sec

There were no significant differences in the maturation rates among the replicates (range: 71.6 ± 6.4% to 78.8 ± 7.2%). Irrespective of the treatment, normal female and male pronuclear formation was seen in more than 96% of the oocytes fertilized, and the percentage of oocytes with additional sperm heads was lower than 6%. Penetration and monospermy rates, the number of spermatozoa per oocyte penetrated, the final efficiency of IVF and the cleavage rates were not influenced by the different treatments (Table 2). However, oocytes exposed to H342 and UV irradiation for 5 sec showed a lower (P < 0.02) rate of blastocyst formation (15.2 ± 4.5%) than oocytes from the other groups (range: 26.1 ± 4.5% to 30.7 ± 4.5%) (Fig. 2). No differences were observed in the blastocyst total cell number among the groups.

4. Discussion

In the present study, we evaluated the effects of H342 and UV irradiation, separately and in combination, on the fertilization and subsequent development of matured porcine oocytes. For that, we used a standard procedure for IVF routinely employed in our laboratory (control group) with which penetration, monospermy,
cleavage and blastocyst rates were within expected normal ranges in the two experiments.

Our results demonstrated that the exposure of matured oocytes to 5 μg/mL H342 for 12 min without subsequent UV irradiation did not interfere with fertilization or with development to the blastocyst stage. These results were consistent in the two experiments performed and confirmed previous reports in which a 30-min exposure to 5 μg/mL H342 did not affect the rate of development to the blastocyst stage of parthenogenetically activated porcine oocytes [25]. However, it has been demonstrated that the treatment of mouse zygotes with H342 at higher concentrations (7.5 and 10 μg/mL) for only 3 min significantly inhibited their development into blastocysts [26]. Moreover, the exposure of porcine oocytes to 5 μg/mL H342 for 2 h reduced embryo development and the quality of blastocysts formed and increased the rate of degenerated embryos [25]. These results suggest that the concentration of the stain and/or the length of the exposure to the stain could play an important role in oocyte and embryo viability. We used the stain concentration (5 μg/mL) and exposure time (<30 min) usually employed for enucleation [3,5–7,9–11]. These conditions are sufficient for giving fluorescence to the chromosomes at metaphase and did not compromise the functionality of the oocytes.

Because chromosomes at metaphase can be observed under UV irradiation when they are stained with H342, little attention has been paid to the individual effect of UV light on the developmental ability of mature oocytes. Tsunoda et al [26] reported that exposure to only UV radiation for 5 to 60 sec did not inhibit the development of mouse eggs to blastocysts. In agreement with these results, we did not find deleterious effects on fertilization parameters, embryo development or the quality of the blastocysts when mature oocytes were UV irradiated for 5 or 30 sec without previous H342 staining. However, this is in contrast to the results reported using bovine oocytes. Dominko et al [27] stated, as unpublished data, that when bovine oocytes were irradiated with UV in the absence of H342, the developmental potential was compromised. The reasons for this discrepancy are not clear but could be due to differences in the species and methods used.

It is generally assumed that H342 dye in combination with UV irradiation can induce detrimental effects on oocytes and their subsequent development capacity. However, to the best of our knowledge, no studies to date have investigated such effects on porcine oocytes.
Table 2

Effect of Hoechst 33342 (H342) staining and ultraviolet (UV) irradiation for 5 sec on the fertilization parameters of mature oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. oocytes</th>
<th>Penetration %</th>
<th>Monospermy %</th>
<th>EO</th>
<th>Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>H342</td>
<td>UV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– –</td>
<td>185</td>
<td>77.2 ± 5.0</td>
<td>57.4 ± 4.6</td>
<td>1.5 ± 0.1</td>
<td>38.1 ± 3.7</td>
</tr>
<tr>
<td>+ –</td>
<td>194</td>
<td>77.9 ± 4.9</td>
<td>59.2 ± 4.6</td>
<td>1.5 ± 0.1</td>
<td>39.8 ± 3.7</td>
</tr>
<tr>
<td>+ +</td>
<td>172</td>
<td>74.8 ± 5.1</td>
<td>52.2 ± 4.8</td>
<td>1.5 ± 0.1</td>
<td>34.9 ± 3.8</td>
</tr>
<tr>
<td>– +</td>
<td>198</td>
<td>78.6 ± 4.9</td>
<td>59.1 ± 4.5</td>
<td>1.5 ± 0.1</td>
<td>40.5 ± 3.7</td>
</tr>
</tbody>
</table>

Penetration: number of oocytes penetrated/total inseminated; monospermy: number of oocytes containing only one sperm head or one male pronucleus/total penetrated; EO: number of spermatozoa/oocyte penetrated; efficiency: number of monospermic oocytes/total inseminated.

The present results indicate a clear deleterious effect on embryo development when mature oocytes were simultaneously exposed to H342 and UV irradiation. Because no effects on embryo development were observed when mature oocytes were separately exposed to H342 and UV irradiation, the toxicity of the procedure might be due to a synergistic effect from the interaction of both factors. The deleterious effects increased with the length of exposure to UV light. Sperm penetration and monospermy were lower in oocytes exposed to H342 and UV irradiation for 30 sec than those of the control group, and as a result the efficiency of fertilization was also lower. Interestingly, H342-pretreated oocytes and UV irradiated for 30 sec showed positive relationship between penetration and monospermy rates (lower penetration and lower monospermy). In porcine IVF programs, it is generally accepted that a decreased penetration rate is usually associated with an increased rate of monospermy. In our study, a higher proportion of oocytes stained and irradiated for 30 sec was inefficient to be penetrated and, in parallel, a higher proportion of penetrated oocytes had insufficient ability to block polyspermy. We can speculate that this might be due to a delayed or incomplete zona reaction and/or to a damaged and imperfect zona caused by the combined exposition to H342 and UV irradiation. Cleavage rates at 48 h postinsemination and blastocyst formation were strongly decreased in H342-pretreated oocytes exposed to 30 sec of UV irradiation compared with those of the other groups. These results were not unexpected because previous reports have indicated that the exposure of H342-stained oocytes to UV irradiation for 30 sec produced changes in the protein synthesis of bovine oocytes [17], and it decreased viability in rabbit oocytes [15]. Moreover, UV exposure of mouse zygotes for 20 to 30 sec inhibited the development to the blastocyst stage [26]. However, it has been shown that the exposure of H342-pretreated oocytes to UV irradiation for less than 10 to 15 sec did not affect the developmental potential in rabbit [15] and bovine [16] reconstructed embryos. These findings are in contrast with the present results, as UV irradiation for only 5 sec of H342-treated oocytes did decrease their developmental competence, although fertilization parameters and cleavage rates were not affected. Deleterious effects of very short (3 sec) UV irradiation periods have also been reported on the in vitro maturation of goat oocytes stained with H342 [18]. It seems likely that the differences among the species and/or in the kind of embryo cultured (i.e., zygote, reconstructed, parthenote) could be the cause of these discrepancies.

The harmful effects of exposure to H342 and UV irradiation on the embryonic development of oocytes could be due primarily to nuclear DNA damage. It is known that irradiation of the metaphase plate with short-wavelength (254 nm) UV light produces structural abnormalities in the female pronuclei of pig oocytes, including the presence of multiple micronuclei of different sizes with condensation and the dispersal of DNA fragments [28], and it prevents female pronuclear formation in bovine oocytes at fertilization [29]. Moreover, focused UV-A irradiation of the metaphase plate of mature bovine oocytes with a longer wavelength (>330 nm) induced abnormal female pronucleus formation at fertilization [29]. However, in our study, which used the recommended wavelength (350 nm) of UV light to excite the H342 dye, no effects were observed in the percentage of oocytes forming female pronuclei or in pronuclear morphology and chromatin configuration. The exposure time of the oocytes to UV irradiation (5 and 30 sec) did not modify that pattern. That discrepancy might be explained by species-specific differences in the sensitivity to UV irradiation in the same way that oocytes and embryos of different species must have different sensitivities to cool white fluorescent light [30]. A second explanation for the differences in female pronuclear formation between both studies is that the deleterious effects of H342 in combination with UV irradiation on porcine oocytes may go unperceived during the fertilization period but
then be manifested during later development stages. Little attention has been paid to the effects of H342 and UV irradiation on male pronuclear formation after fertilization of selective metaphase II-irradiated bovine oocytes [29]. In our study, the percentage of oocytes forming male pronuclei, the number of male pronuclei formed in each oocyte, the male pronuclear morphology and the chromatin configuration were independent of whether the oocytes were exposed to H342 followed by UV irradiation. The fact that male pronuclear formation was not affected after oocyte non-selective UV irradiation suggests that the cytoplasmic components of the oocyte-modulating sperm decondensation and male pronuclear formation are not compromised by the combined exposure to H342 and UV irradiation.

It could be speculated that nuclear DNA damage should not represent a concern because nuclear DNA is removed during oocyte enucleation and that results from IVF embryos may not totally reflect the potential effect (toxicity) of H342/UV on the development of nuclear transfer embryos. However, additional factors could be implicated in the negative effects of H342 and UV irradiation on oocyte development. First, the excess of H342 in the cytoplasm can be transferred to the nucleus of the reconstructed oocytes in the same way that the fluorochrome is transferred from H342-stained sperm to the female pronucleus or corona cells [31]. Even if that were the case, it might not be problematic because reconstructed oocytes are not irradiated again, and H342 itself, at the doses usually used for enucleation, does not produce negative effects on embryonic development, as demonstrated in our study. Second, when an oocyte pretreated with H342 is UV irradiated, not only the nucleus but also the cytoplasmic organelles are exposed to the effects of the treatment. Therefore, the direct effect of H342/UV exposure on cytoplasmic organelles must be considered. H342 strongly binds to the minor groove of DNA, preferentially to AT-rich regions [13]. Mitochondrial DNA possesses many AT-rich sites in different genes coding for electron transport [32]. Recently, it has been demonstrated that H342 has the ability to bind strongly with mitochondrial DNA, leading to radiosensitization in human malignant glioma cells [33] and resulting in the accumulation of reactive oxygen species (ROSs), such as H₂O₂, which in turn alter the mitochondrial membrane potential, leading to cell death [32]. If a similar mechanism occurs in the mitochondria of the oocyte after exposure to H342 and UV irradiation, the embryonic development might be impaired as a consequence of ROS accumulation inside of the cell. Further studies are in progress.

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**Fig. 2.** Effect of Hoechst 33342 (H342) and ultraviolet (UV) irradiation for 5 sec on the preimplantational development of in vitro-fertilized porcine oocytes. The total numbers of putative zygotes cultured in each group are given in parentheses. After maturation, oocytes were incubated for 12 min in maturation medium with or without 5 µg/mL H342 and then individually allocated into 20-µL drops of maturation medium overlaid with warm mineral oil to be exposed or not exposed to UV irradiation, according to the experimental design. **a** Different superscripts indicate differences (P < 0.02, at least). Cleavage rate: percentage of embryos cleaved/total oocytes inseminated; blastocyst rate: percentage of blastocysts/total oocytes inseminated. The number of cells was evaluated in 7-day-old blastocysts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H342</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>n</td>
<td>(269)</td>
<td>(269)</td>
</tr>
</tbody>
</table>

100 80 60 40 20 0
Cleavage (%)

40 30 20 10 0
Blastocyst (%)

50 40 30 20 10 0
Number of total cells
to evaluate the mitochondrial functionality of oocytes after exposure to H342 and UV irradiation.

In conclusion, our results demonstrate that separate exposure of the oocytes to H342 or UV irradiation does not interfere with their fertilization and developmental potential. In contrast, H342 combined with UV irradiation has deleterious effects on the development of in vitro-fertilized porcine oocytes, with the effects being more drastic with increased exposure to UV irradiation. These results advise that H342 staining combined with UV irradiation should be avoided in recipient porcine oocytes used for nuclear transfer.

Acknowledgments

The authors acknowledge the technical support of S. Gomez. This study was supported by the Seneca Foundation of Murcia (GERM 04543/07) and MICINN (AGL2009-12091). The Seneca Foundation of Murcia is acknowledged for its grant-based support of C. Maside (07875/BPS/07).

References

[26] Tsunoda Y, Shiota Y, Onodera M, Nakamura K, Uchida T. Differential sensitivity of mouse pronuclei and zygote cyto-


ARTICLE 2

Effects of Hoechst 33342 staining and ultraviolet irradiation on mitochondrial distribution and DNA copy number in porcine oocytes and preimplantation embryos
Effects of Hoechst 33342 Staining and Ultraviolet Irradiation on Mitochondrial Distribution and DNA Copy Number in Porcine Oocytes and Preimplantation Embryos

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SUMMARY

Hoechst 33342 (H342), in combination with ultraviolet (UV) irradiation, is frequently used to aid or confirm the enucleation of porcine oocytes in somatic cell nuclear transfer programs. The exposure of oocytes to H342 and UV irradiation has a deleterious effect on the development of in vitro-fertilized porcine oocytes, with increasing exposure to UV irradiation (up to 30 sec) having more drastic effects. It has been hypothesized that this decrease in embryonic development could be due to damage to the mitochondrial DNA (mtDNA). To investigate this hypothesis, we analyzed the mitochondrial distribution and DNA copy number of in vitro-matured porcine oocytes exposed to H342/UV and the subsequent embryonic development compared with the mitochondrial distribution and DNA copy number of in vivo-derived oocytes and embryos. Using quantitative, real-time polymerase chain reaction (qPCR) protocols to analyze mtDNA and confocal laser scanning microscopy with MitoTracker Deep Red to determine mitochondrial distribution, we demonstrated that the simultaneous exposure of in vitro-matured porcine oocytes to H342 staining and UV irradiation is associated with reduced oocyte developmental competence and abnormal mitochondrial distribution in the resulting cleaved embryos. In addition, 2- to 4-cell embryos derived from oocytes exposed to H342/UV showed a significant decrease in mtDNA copy number. These results should be considered when H342/UV procedure is used during nuclear transfer in recipient porcine oocytes.


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INTRODUCTION

Pig cloning by somatic cell nuclear transfer (SCNT) has become attractive in recent years because of its physiological resemblance to humans. Pigs can be used as research models and for creating genetically modified animals. The number of cloned pigs has continuously increased since the first piglets were produced by SCNT in 2000 (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), but this method still has a low overall efficiency (reviewed by Vajta et al., 2007). Success of the SCNT technique depends on a number of factors, and enucleation of a recipient oocyte is particularly critical to cloning efficiency. Porcine oocytes are especially difficult to enucleate because their cytoplasm contains many lipid droplets. Ultraviolet (UV) irradiation and excitable DNA-specific dyes such as Hoechst 33342 (H342) (Abbreviations: H342, Hoechst 33342; IVF, in vitro fertilization; mtDNA, mitochondrial DNA; UV, ultraviolet.)
TABLE 1. Effect of the Exposure of In Vitro-Matured Porcine Oocytes to Hoechst 33342 (H342) Staining and Ultraviolet (UV) Irradiation on Their Developmental Competence

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oocytes (n)</th>
<th>Cleavage (n%)</th>
<th>Blastocyst (n%)</th>
<th>No. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>586</td>
<td>365/62.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>143/24.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.1 ± 1.1</td>
</tr>
<tr>
<td>H342/UV</td>
<td>489</td>
<td>200/40.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52/10.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.8 ± 1.8</td>
</tr>
</tbody>
</table>

Cleavage: number of embryos cleaved/total oocytes fertilized; blastocyst: number of blastocyst/total oocytes fertilized. Different letters (a, b) within the same column represent a significant difference ($P < 0.0001$).

have often been used to aid the visualization of the nuclear material and the polar body before enucleation (Estrada et al., 2008; Lee et al., 2008; Hickey et al., 2011) or to confirm the enucleation of recipient oocytes after manipulation (Krath et al., 2005; Das et al., 2010; Koo et al., 2010; Biswas et al., 2011). Exposure of the recipient oocytes to H342 and UV irradiation, however, may be detrimental for oocyte developmental competence, as reported for several species including cattle (Smith, 1993; Dominko et al., 2000), goats (Vellilla et al., 2002), rabbits (Yang et al., 1990), and mice (Versieren et al., 2010). We have recently demonstrated that the exposure of oocytes to H342 and UV irradiation has a deleterious effect on the development of in vitro-fertilized porcine oocytes, with increasing exposure to UV irradiation (up to 30 sec) producing more drastic effects (Maside et al., 2011). According to Dominko et al. (2000), the negative effects of H342 and UV irradiation on oocyte development could link to damaged effects on nuclear DNA and/or maternal cytoplasmic organelles and proteins. Nuclear DNA damage should not be a concern because nuclear DNA is removed during oocyte enucleation; the effect of H342/UV exposure on cytoplasmic organelles, however, must be considered.

H342 strongly binds to the minor groove of DNA, particularly to AT-rich regions (Robinson et al., 1998). Mitochondrial DNA (mtDNA) possesses many AT-rich sites (Athar et al., 2011). Recently, it has been demonstrated that H342 can strongly bind to mtDNA and induce an increase in reactive oxygen species (ROS) in human malignant glioma cells, leading to cell death (Athar et al., 2010). If a similar mechanism occurs in the mitochondria of the oocyte after exposure to H342 and UV irradiation, embryonic development might be impaired as a consequence of ROS accumulation inside of the cell. Because mitochondrial function, activity, and distribution are vital elements of normal fertilization and developmental viability for embryos, the objective of the present study was to evaluate the effects of H342 staining and UV irradiation on mitochondrial distribution and DNA copy number in developing porcine oocytes and pre-implantation embryos.

RESULTS
Effect of H342 and UV Irradiation on the Development of In Vitro-Fertilized Oocytes

A total of 1,075 in vitro-matured oocytes were fertilized and cultured for 7 days to evaluate embryo development. As shown in Table 1, oocytes exposed to H342/UV showed a lower ($P < 0.001$) cleavage rate (40.9%) and blastocyst formation (10.6%) than oocytes from the control group (62.3% and 24.4%, respectively). No differences were observed in blastocyst total cell number between groups.

Effects of H342 and UV Irradiation on the Distribution of Active Mitochondria in Mature Oocytes and Preimplantation Embryos

At the time of collection, most (11/13; 84.6%) of the in vivo-matured oocytes contained active mitochondria distributed throughout the cytoplasm, although these organelles were more abundant in the peripheral cytoplasm than in the inner cytoplasm (also see Table 2). Strong, active-mitochondrial staining was commonly observed in the first polar bodies. This distribution of active mitochondria was also present in 90.9% of the in vitro-matured control

TABLE 2. Mitochondrial Distribution in Oocytes and Embryos Derived From Oocytes Exposed to Hoechst 33342 (H342) Staining and Ultraviolet (UV) Irradiation

<table>
<thead>
<tr>
<th></th>
<th>Mature Oocytes</th>
<th>Zygotes</th>
<th>2-to-4 cells</th>
<th>Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Normal (%)</td>
<td>N Normal (%)</td>
<td>N Normal (%)</td>
<td>N Normal (%)</td>
</tr>
<tr>
<td>In vivo</td>
<td>13 (84.6)</td>
<td>18 (100)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26 (100)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15 (100)</td>
</tr>
<tr>
<td>In vitro control</td>
<td>22 (90.9)</td>
<td>8 (50.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27 (86.9)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19 (100)</td>
</tr>
<tr>
<td>In vitro H342/UV</td>
<td>19 (88.4)</td>
<td>12 (75.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30 (75.0)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11 (100)</td>
</tr>
</tbody>
</table>

Normal mitochondrial distribution pattern in: mature oocytes, distributed throughout the cytoplasm and more abundant in the peripheral cytoplasm than in the inner cytoplasm; zygotes, aggregations associated with the pronuclei in the inner cytoplasm and near the plasma membrane; 2-to-4-cell embryos, distributed homogeneously in the inner cytoplasm, mainly around the periphery of each blastomere and aggregated in the nuclear region; blastocysts, distributed homogeneously in both the inner cell mass and trophectoderm cells. Chi-square test with Yates correction for continuity.

<sup>a,b</sup>$P < 0.002$;<sup>c</sup>$P < 0.001$.
oocytes and in 68.4% of the in vitro-matured oocytes exposed to H342/UV (20/22 and 13/19, respectively; Fig. 1). Oocytes with poor (1/13, 1/22, and 4/19 for in vivo-, in vitro-control, and in vitro-H342/UV samples, respectively) or no staining for mitochondria (1/13, 1/22, and 2/19 for in vivo-, in vitro-control, and in vitro-H342/UV, respectively) were considered to be mature oocytes with abnormal mitochondrial distribution.

After fertilization, 100% of the in vivo zygotes (18/18) showed active mitochondrial aggregates associated with the pronuclei in the inner cytoplasm and near the plasma membrane. There was also strong mitochondrial staining in

![Confocal micrographs of mature porcine oocyte obtained in vivo (A, A’) and produced in vitro from oocytes non-exposed (B, B’) or exposed to H342 staining and UV irradiation (C, C’). Confocal images show the distribution of active mitochondria stained with MitoTracker Deep Red 633. Red channel of an equatorial section through the polar body (A–C) and superposition of the transmission image and red confocal channel of an equatorial section through the polar body (A’–C’) are shown. Active mitochondria are observed throughout the cytoplasm and are more abundant in the peripheral cytoplasm. Strong active mitochondrial staining is observed in the polar body (A–C). Bar, 40 μm.](image-url)
the first and second polar bodies. This pattern was observed in 75% (6/8) and 58.3% (7/12) of the in vitro, monospermic zygote controls and in those exposed to H342/UV, respectively (Fig. 2A–C). In vitro zygotes with no mitochondria around of the pronuclei were considered to be zygotes with abnormal mitochondrial distribution (Fig. 2D).

During early cleavage, active mitochondria were distributed homogeneously in the inner cytoplasm, mainly around the periphery of each blastomere, and aggregated in the nuclear region in most of in vivo (25/26; 96.2%) and in vitro control (24/27; 88.9%) 2- to 4-cell embryos (Fig. 3A,B). In contrast with the 2- to 4-cell in vivo- and in vitro-control embryos, only 13/30 (43.3%) of the 2- to 4-cell embryos obtained from the oocytes exposed to H342/UV showed that distribution. Additionally, 20% (6/30) of the embryos in the exposed group showed active mitochondria with strong staining in anucleate cell fragments (Fig. 3C) and 36.6% (11/30) presented blastomeres with poor or no staining for mitochondria (Fig. 3D).

Active mitochondria were distributed homogeneously in both the inner cell mass and trophoectoderm cells in all blastocysts analyzed, irrespective of the experimental group (Fig. 4).

**Effects of H342 and UV Irradiation on mtDNA Copy Number in Mature Oocytes and Preimplantation Embryos**

The number of mtDNA copies per mature oocyte, zygote, 2- to 4-cell embryo, and blastocyst obtained in vivo and in vitro from oocytes exposed or non-exposed to H342/UV are shown in Figure 5. There were no significant differences in the mtDNA copy number among in vivo, in vitro-control, and in vitro-exposed mature oocytes (range from 347.023 ± 46.334 to 400.615 ± 89.487). The mtDNA copy numbers of the embryos obtained from the oocytes exposed to H342/UV, however, were lower (P < 0.04) for zygotes (85.909 ± 19.503) and 2- to 4-cell embryos (110.280 ± 23.082) and higher (P < 0.02) for blastocysts (557.115 ± 176.717) compared to quantities obtained from in vitro-derived embryos (162.324 ± 25.508; 180.878 ± 17.009; and 167.287 ± 33.379; respectively). No differences were observed among in vivo- and in vitro-control oocytes and embryos.

When comparisons were made within each experimental group (in vivo or in vitro, exposed or non-exposed to H342/UV), significant differences were found according to developmental stage (Fig. 5). In the in vivo group, mature oocytes contained an average of 400.615 ± 89.487 mtDNA copies, which was higher (P < 0.02) than in zygotes, 2- to 4-cell embryos, and blastocysts (162.324 ± 25.508; 180.878 ± 17.009; and 167.287 ± 33.379; respectively). In the in vitro groups, the average mtDNA copy number was 347.023 ± 46.334 and 370.441 ± 77.126 for in vitro-control oocytes and oocytes exposed to H342/UV, respectively; this value was lower (P < 0.02) in zygotes (100.575 ± 20.995 and 85.909 ± 19.503; respectively) and 2- to 4-cell embryos (176.192 ± 26.007 and 110.280 ± 23.082; respectively). Nevertheless, mtDNA copy number in blastocysts increased to 320.056 ± 52.590 and 557.115 ± 176.717 for the control and H342/UV groups, respectively; these numbers did not differ significantly from those of mature oocytes.

**DISCUSSION**

We previously demonstrated that the combination of H342 staining with UV irradiation has clear, deleterious effects on the fertilization and subsequent development of in vitro-matured porcine oocytes. (Maside et al., 2011). In the present study, cleavage rate and blastocyst formation were again significantly lower in oocytes exposed to H342/UV than in oocytes from the non-exposed group.

It has been hypothesized that this decrease in embryonic development could be due to mtDNA damage. The proper positioning of mitochondria in the oocyte is necessary to provide ATP and calcium to specific regions, thereby supporting normal developmental processes (Sun et al., 2001; Krischer, 2004; Torner et al., 2004; Katayama et al., 2006). In the present study, the distribution of mitochondria in oocytes and embryos was characterized by distinct localized aggregation patterns. In most in vivo- and in vitro-control matured oocytes, the mitochondria were distributed throughout the cytoplasm, although more abundant in the periphery, with strong staining in the first polar body (85% and 90%, respectively). This finding is consistent with previous reports from several species, including porcine (Sun et al., 2001; Katayama et al., 2006; Romek et al., 2011), bovine (Hytte et al., 1986; Stojkovic et al., 2001), mouse (Tokura et al., 1993), and hamster (Barnett et al., 1996). Exposing oocytes to H342/UV, however, disrupted this pattern and the number of oocytes with this mitochondrial pattern tended to decrease (P = 0.15) compared to the non-exposed, matured oocytes. It should be noted that the oocytes were processed for analysis only 1 hr after exposure to H342/UV. The possible effect produced by the staining and the irradiation in 1 hr is difficult to explain. It is possible that the exposure conditions used in the present study accelerated aging in some oocytes since aging is thought to be a process of deterioration in the quality of mature oocytes that may lead to mitochondrial dysfunction, as has been demonstrated in humans (Wilding et al., 2001) and pigs (Hao et al., 2009), which could decrease ATP and increase ROS production (Cui et al., 2011), resulting in a subsequent negative effect on embryo development. This phenomenon could explain the lower embryo development rate obtained in our study after in vitro fertilization (IVF) of these oocytes.

The present study reveals that 100% of in vivo zygotes showed active mitochondrial aggregations associated with the pronuclei and the first and second polar bodies. Peripheral clustering and clustering at the periphery of each blastomere constituted the most marked distribution in in vivo 2- to 4-cell embryos (96%). In contrast, a significantly lower number of zygotes and 2- to 4-cell embryos derived from oocytes exposed to H342/UV showed a normal mitochondrial pattern. Although differences between in vivo and in vitro mitochondrial distribution may be caused by
Figure 2. Confocal images of equatorial sections that illustrate the distribution of active mitochondria in in vivo-derived zygotes (A, A’, and A’’) and in in vitro-produced zygotes from oocytes non-exposed (B, B’ and B’’) or exposed to H342 staining and UV irradiation (C, C’, and C’’). Zygotes were stained with MitoTracker Deep Red 633 to visualize active mitochondria (red) and with H342 to stain pronuclei and polar bodies (blue). A–D: Red confocal channel; A’–D’ superposition of red and blue confocal channels; and A’’–D’’ superposition of red, blue, and transmission confocal channels are shown. Active mitochondria are associated with the pronuclei, the inner cytoplasm, areas close to the plasma membrane, and areas around the polar bodies (A–C’’). Figures D, D’, and D’’ show a zygote with abnormal mitochondrial distribution (active mitochondrial staining is only observed in the polar bodies). Bar, 40 μm.
Figure 3. Confocal images of equatorial sections that illustrate the distribution of active mitochondria in porcine 2- to 4-cell embryos. Embryos were stained with MitoTracker Deep Red 633 to visualize active mitochondria (red) and with H342 to stain cell nuclei and polar bodies (blue). A–C: Red confocal channel; (A–C') superposition of red and blue confocal channels; and (A''–C'') superposition of red, blue, and transmission confocal channels are shown. Active mitochondria are distributed throughout the inner cytoplasm, mainly in the periphery of each blastomere, and aggregated in the nuclear region in in vivo-derived 2- to 4-cell embryos (A, A', and A'') and in in vitro-derived 2- to 4-cell embryos (B, B', and B''). Four-cell embryo from the in vitro-exposed (H342/UV) group (C, C', and C'') exhibiting an enucleate blastomere with active mitochondria (asterisk) and an enucleate cell fragment showing strong MitoTracker Deep Red 633 staining (arrow head). Two-cell embryo from the in vitro-exposed (H342/UV) group (D, D', and D'') showing active mitochondria in one blastomere and the absence of active mitochondria in the other. Note the strong mitochondrial staining around the polar body. Bar, 40 μm.
suboptimal in vitro culture conditions (Sun et al., 2001), the differences observed between in vitro 2- to 4-cell controls and those derived from exposed oocytes were clearly due to H342/UV-induced damage.

It is noteworthy that most of the embryos cleaved from oocytes exposed to H342/UV did not present mitochondrial aggregation around nuclei. Perinuclear aggregation of mitochondria is positively correlated with the developmental...
capacity of fertilized porcine embryos (Katayama et al., 2006), possibly providing a local concentration of energy for nucleo-cytoplasmic mRNA transport (Prachar, 2003). This aggregation may be a criterion that can predict the developmental potential of embryos. In contrast to in vivo and in vitro controls, two disproportionate patterns of mitochondrial distribution were found in 2- to 4-cell embryos derived from oocytes exposed to H342/UV. The first pattern was characterized by the presence of blastomers with poor or no staining for mitochondria (36%). During early cleavage, blastomers with a smaller population of mitochondria that are competent to produce ATP may become dysfunctional and fragment (Cummins, 2004) or die during subsequent culture (Van Blerkom, 2000). The second pattern consisted of strong staining in anucleate cytoplasmic fragments (20%). Cellular fragmentation is also indicative of poor embryo quality (Lindner and Wright, 1983; Puissant et al., 1987; Mateusen et al., 2005), resulting in a compromising effect on embryo cleavage (Antczak and Van Blerkom, 1999). According to Hao et al. (2003), cytoplasmic fragmentation is associated with porcine IVF embryos and nuclear transfer embryos undergoing apoptosis. In the current study, the percentage of embryo fragmentation in the in vitro-control group (4%) was not comparable to the percentage detected in the exposed group (20%). Thus, the inappropriate distribution of mitochondria in cleaved embryos could be the cause, at least in part, of the low number of blastocysts formed in the H342/UV exposed group. Interestingly the quality of the blastocyst, in terms of mitochondrial distribution, was not affected. Future research should focus on determining the implantation ability of these blastocysts.

Mitochondrial function is also strongly associated with mtDNA copy number. Although there is evidence that a minimum number of mtDNA copies in oocytes or embryos is necessary during pre- and post-implantation embryo development (Dumollard et al., 2007; Wai et al., 2010), little is known about mtDNA copy number in pig oocytes and embryos. The mean mtDNA copy number per in vitro- or in vivo-matured pig oocyte reported here is comparable to the number determined by other authors in bovine (Tamaia et al., 2004; May-Panloup et al., 2005), porcine (Spinkings et al., 2007), human (Chen et al., 1995; Steuerwald et al., 2000; Reynier et al., 2001; Barratt et al., 2002), and mouse oocytes (Steuerwald et al., 2000). Following fertilization, mtDNA copy number declined by about 60% in both the in vivo- and in vitro-control groups. These findings agree with those reported for cows (May-Panloup et al., 2005) and pigs (Spinkings et al., 2007) with IVF embryos, in which the mtDNA content was reduced after fertilization. The drastic reduction of mtDNA after fertilization may be due to active destruction of mtDNA molecules (May-Panloup et al., 2005) during the proteasome-mediated destruction of paternal mtDNA (Sutovsky et al., 2000). If the destruction of mtDNA occurs, it was more drastic in in vitro-derived embryos from oocytes exposed to H342 and UV irradiation: the mtDNA copy number declined by about 80% after fertilization, with values significantly lower than those observed in in vivo zygotes and 2- to 4-cell embryos. This suggests that in the embryos derived from the H342/UV exposed oocytes, mtDNA copy number per blastomere decreased more after each embryonic cell division than in the in vivo-derived embryos. These blastomers will then progressively lose their capacity to generate ATP through oxidative phosphorylation as they become more reliant on anaerobic respiration (St John et al., 2010).

In contrast to the in vivo model provided here, in which mtDNA was constant for zygotes, 2- to 4-cell, and blastocyst stage embryos, the mtDNA content of both in vitro groups increased drastically in blastocysts. This finding was expected considering the previous studies on bovine and porcine IVF embryos (May-Panloup et al., 2005; Spinkings et al., 2007). This increase in mtDNA copy number in in vitro embryos can be interpreted as an accelerated mtDNA replication rate, similar to that reported in rat oocytes by Kameyama et al. (2007), who suggested that the in vitro culture system can perturb mitochondrial transcription and the regulation of mtDNA replication.

In conclusion, this report demonstrates that the simultaneous exposure of mature porcine oocytes to H342 staining and UV irradiation is associated with reduced oocyte developmental competence and abnormal mitochondrial distribution pattern and with reduced mtDNA copy number in the resulting cleavage embryos. Such outcomes have to be considered when H342 and UV irradiation are used to aid or confirm enucleation for nuclear transfer.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma–Aldrich Chemical Company (Alcobendas, Madrid, Spain) unless otherwise indicated.
In Vitro Maturation and Fertilization of Oocytes and In Vitro Culture of Early Embryos

**Culture media** Dulbecco's phosphate-buffered saline medium was used for the collection of cumulus–oocyte complexes (COCs) and for washing; this medium is composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, and 1.46 mM CaCl₂-2H₂O and was supplemented with 4 mg/ml bovine serum albumin (BSA: fraction V), 0.34 mM sodium pyruvate, 5.4 mM glucose, and 70 μg/ml kanamycin (inDPBS). The oocyte maturation medium was BSA-free North Carolina State University medium (NCSU-23; Petters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid, 0.8 mM cysteine, 10 ng/ml epidermal growth factor, and 5 mM 9-cis retinoic acid. The basic medium used for fertilization was essentially the same as that used by Abeydeera and Day (1997). This medium, a modified Tris-buffered medium (mTEM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris (crystallized free base), 11 mM glucose, and 5 mM sodium pyruvate, and was supplemented with 0.5 mM caffeine and 0.2% BSA. The embryo culture medium was a sequential medium based on NCSU-23 and supplemented with 0.4% BSA.

**Oocyte collection and in vitro maturation** Ovaries were obtained from prepubertal gilts in a local slaughterhouse and transported to the laboratory at 35°C within 1 hr after collection in 0.9% NaCl containing 70 μg/ml kanamycin. COCs were aspirated from medium-sized follicles (3–6 mm in diameter) using an 18-gauge needle connected to a 10-ml disposable syringe. Oocytes with a compact cumulus mass and dark, evenly granulated cytoplasm were washed three times in maturation medium, and 70–80 oocytes were transferred into each well of a 4-well multi-dish (Nunc, Roskilde, Denmark) containing 500 μl of maturation medium supplemented with 10 IU/ml of equine chorionic gonadotrophin (eCG, Folligon, Intervet International B.V., Boxmeer, The Netherlands) and 10 IU/ml of human chorionic gonadotrophin (hCG, Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain). The oocytes were cultured in these wells for 20–22 hr, and were then incubated for another 20–22 hr in maturation medium without hormones. Oocyte maturation was carried out under mineral oil at 39°C in a humidified atmosphere of 5% CO₂ in air. After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium by vortexing for 2 min at 1,660 rounds/min and were washed twice in maturation medium.

**Staining with H342 and ultraviolet irradiation** Immediately after maturation, groups of 30 oocytes were incubated for 12 min in 500 μl of maturation medium containing 5 μg/ml H342. Subsequently, oocytes were washed in maturation medium and individually allocated into 20-μl drops of maturation medium overlaid with warm mineral oil. Each oocyte was rotated with the help of two micromanipulators (Eppendorf, Hamburg, Germany) to visualize the first polar body, and only intact oocytes that had extruded the first polar body were selected as "mature oocytes." Mature oocytes were individually exposed to UV irradiation for 30 sec under a mercury short arc lamp with a UVA filter (330–380 nm excitation).

**In vitro fertilization and in vitro culture** In vitro fertilization was performed as described by Gil et al. (2003). Briefly, matured oocytes were washed three times in pre-equilibrated fertilization medium, and groups of 30 denuded oocytes were placed in 50-μl drops of the same medium in a 35 mm × 10 mm Petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) under mineral oil. The oocytes were held at 39°C in an atmosphere of 5% CO₂ in air for about 30 min until spermatozoa were added. Semen from a mature Pietrain boar was processed and cryopreserved in 0.5-ml straws, as described by Roca et al. (2004). For each replicate, one pool of semen was made from two straws thawed in a circulating water bath at 37°C for 20 sec. Next, 100 μl of thawed semen was washed three times by centrifugation at 1,900g for 3 min in mDPBS. The resulting pellet was resuspended in fertilization medium, and 50 μl of this sperm suspension containing 3 × 10⁵ spermatozoa were added to a 50-μl drop of fertilization medium containing the oocytes. The spermatozoa:oocyte ratio was 1,000:1. The gametes were co-incubated at 39°C in a humidified atmosphere of 5% CO₂ in air for 5 hr.

After IVF, each presumptive zygote was rotated with a thin pipette to visualize the polar bodies, and only zygotes with a single cell and two visible polar bodies were used in the experiments. Zygotes were washed three times in pre-equilibrated embryo culture medium. Subsequently, they were transferred to a 4-well multi-dish (30 zygotes per well), with each well containing 500 μl of the same medium under mineral oil. The zygotes were cultured at 39°C in a humidified atmosphere of 5% CO₂ in air for 7 days to assess embryo development. During the first 2 days (Day 0 = day of fertilization) of culture, glucose in the basic embryo culture medium was replaced with 0.33 mM sodium pyruvate and 4.5 mM sodium lactate. Then, all embryos were removed and cultured in fresh NCSU-23 medium containing 5.5 mM glucose.

At 2 and 7 days after insemination, the cleavage rate (percentage of the number of oocytes divided to 2- to 4-cells/total cultivated) and blastocyst formation (percentage of the number of blastocysts/total cultivated), respectively, were evaluated under a stereomicroscope. An embryo that had cleaved to the two-cell stage or beyond was counted as cleaved, and an embryo with a clear blastocoele was defined as a blastocyst. The total cell number was used as an indicator of embryo quality, and was evaluated by counting each blastocyst on a slide in 4 μl of a dilution of glycerol/DPBS (3:1 vol/vol) containing 10 μg/ml H342, followed by examination using fluorescence microscopy.

**Recovery of In Vivo-Derived Oocytes, Zygotes, 2- to 4-Cell Embryos, and Blastocysts**

**Animals** The experimental protocols were carried out in accordance with the Directive 2010/63/EU EEC for animal
experiments, and were reviewed and approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain. Weaned, crossbred (Landrace × Large White) sows (from two to six parities) were randomly selected for the experiment. Sows were individually held in crates in a mechanically ventilated confinement facility under commercial production conditions (Agropor S.L, Murcia, Spain). Sows were fed a commercial ration twice a day, and water was provided ad libitum.

Detection of estrus and insemination Estrus was detected twice a day, beginning 2 days after weaning, by exposing females to a mature boar and applying manual back-pressure. Females that showed a standing estrus reflex were considered to be in heat. Only sows that exhibited their first standing reflex on the afternoon of the 4th–5th day after weaning were used in this experiment. Sows were artificially inseminated 12 and 24 hr after onset of estrus with doses of semen (3 × 10^9 spermatozoa in 100 ml) from adult Pietrain boars extended with Beltsville Thawing Solution (BTS; Pursel and Johnson, 1975) and kept for a maximum of 24 hr at 17°C.

Embryo collection medium The collection medium was Tyrode’s lactate (TL)-HEPES PVA modified medium (Funahashi et al., 2000) supplemented with 10 mM HEPES (flushing medium).

Collection of samples Sows were subjected to laparotomy, as described by Martinez et al. (2004). On day 1 (Day 0 = onset of estrus), preovulatory follicles (>8 mm in diameter) were aspirated using an 18-gauge needle connected to a 10-ml disposable syringe to obtain mature oocytes. On day 2 of the estrous cycle, oviducts of each animal were flushed with 10 ml of flushing medium to obtain zygotes and 2- to 4-cell embryos. Blastocysts were collected on day 6 of the estrous cycle by flushing the uterine horns with 30 ml of flushing medium. Oocytes and embryos were evaluated under a stereomicroscope at 60× to assess their developmental stage and quality. Only intact oocytes that had extruded the first polar body and fertilized oocytes with a single cell and two visible polar bodies were selected as mature oocytes and zygotes, respectively. Thereafter, oocytes and embryos were washed three times with flushing medium, placed in Eppendorf tubes containing 1.5 ml of the same medium, and transported at 39°C in a thermostatically controlled incubator to our laboratory at the University of Murcia within 2 hr of collection.

Confocal Microscopy of Active Mitochondria In vivo- or in vitro-derived, mature oocytes, zygotes, 2- to 4-cell embryos, and blastocysts were stained with MitoTracker Deep Red 633 (M22426; Molecular Probes, Leiden, The Netherlands) to assess the distribution of active mitochondria. A stock solution of the dye at a concentration of 1 mM was prepared in dimethyl sulfoxide and stored at –20°C. Oocytes and embryos were stained for active mitochondria in maturation or culture medium, respectively, containing 0.5 μM MitoTracker Deep Red for 30 min at 39°C in 5% CO2 in air. Labeled oocytes and embryos were then washed in maturation or culture medium, respectively, three times for 20 min each at 39°C in 5% CO2 in air. After washing, samples were fixed in 4% paraformaldehyde in a 0.1 M sodium cacodylate-buffered solution (pH range 7.2–7.4) for 2 hr at room temperature. Fixed samples were washed three times for 5 min each in phosphate-buffered saline containing 3 mg/ml BSA. After the final wash, oocytes and embryos were mounted on a slide in 4 μl Vectashield (Vector, Burlingame, CA) containing 10 μg/ml H342 for nuclei staining. Samples were observed using a confocal laser scanning microscopy system (Leica TCS SP, Wetzlar, Germany). Fluorochromes were excited with appropriate combinations of excitation and barrier filters; an argon–krypton laser was used for the excitation of MitoTracker Deep Red (644 nm) and an ultraviolet laser was used for the excitation of H342 (354 nm). The images produced by sequential scanning were merged and recorded in a digital format. The images were subsequently displayed using Adobe Photoshop 7 (Adobe Systems, San Jose, CA), and the distribution of active mitochondria was assessed.

DNA Extraction From Oocytes and Embryos and Quantitative Real-Time Polymerase Chain Reaction for mtDNA Analysis DNA was extracted from individual oocytes and embryos using the freeze–thaw method, as previously described by Spikings et al. (2007). Briefly, oocytes, zygotes, 2- to 4-cell embryos, and blastocysts were suspended individually in 50-μl sterile, nuclease-free water. Samples received two freeze–thaw cycles to lyse the oocytes/embryos and release DNA.

To generate external standards a 296-bp fragment of mtDNA (GeneBank accession number NC_012085.1) was amplified by polymerase chain reaction (PCR) as previously described (Spikings et al., 2007) using the following primers (forward primer: 5’-CTC AAC CCT AGC AGA AAC CA; and reverse primer: 5’-TTA GTT GGT GTT CAT GGA ATC G). Then, the PCR product was run on 2% agarose gel and DNA was extracted from the excised bands using QiaQuick Gel Extraction kit (Qiagen, Madrid, Spain) according to the manufacturer’s instructions. The purified PCR product was then sequenced. Both primers and PCR product were verified for specificity using BLAST (http://blast.ncbi.nlm.nih.gov/). It was assumed that 21.73 ng of the 296-bp PCR product contained 6.79 × 10^10 double stranded DNA (PCR product length: 296 bp; concentration: 21.73 ng/μl = 21.73 × 10^-6 g/μl; calculation: (21.73 × 10^-6 g/μl [296 × 660]) × 6.022 × 10^23 = 6.79 × 10^10 molecules/μl). These samples were serially diluted 10-fold in order to construct a standard curve for PCR quantification.

Quantitative real-time PCR (qPCR) analysis was performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The 25 μl qPCR reactions contained 12.5 μl 2X Power SYBR Green
Experimental Design

In this study, the distribution of active mitochondria and the quantification of mtDNA copies were evaluated in vitro and in vivo-derived mature oocytes and embryos. A total of 1,426 in vitro-matured oocytes from three replicates were exposed or non-exposed (in vitro control group) to H342/UV and subjected to IVF with frozen-thawed spermatozoa and in vitro culture. In each replicate, a random subset of the in vitro-matured oocytes, zygotes, 2- to 4-cell embryos, and blastocysts was used to determine the distribution of active mitochondria and the mtDNA copy number, and the remaining were used to evaluate embryo development. In vivo-derived oocytes and embryos (n = 150) were collected from 10 sows in two replicates (2–3 sows/type of sample). For mitochondrial distribution, all samples (oocytes and embryos) were stained and fixed until confocal evaluation. For mtDNA copy number, samples were suspended individually in 50 μl nuclease-free water and frozen at −80°C until assay. qPCR was run in 96-well plates in a total of two replicates. To neutralize variation, each plate contained oocytes and embryos from each source and developmental stage.

Statistical Analysis

All data editing and statistical analyses were performed by SPSS, version 15.0 (SPSS Inc., Chicago, IL, USA). Data concerning the cleavage rate, blastocyst formation, and mitochondrial distribution were expressed in percentages and analyzed using the chi-square test with the Yate’s correction for continuity when necessary. The data concerning the number of cells per blastocyst were evaluated by the Student’s t-test. Data concerning the mtDNA copy number were assessed by analysis of variance (ANOVA) using the MIXED procedure according to a statistical model including the fixed effects of the source of oocytes or embryos (in vivo, in vitro-exposed, or non-exposed to H342/UV groups), within the same developmental stage or the developmental stage within the same experimental group, and the random effect of replicate. When ANOVA showed a significant effect (P < 0.05), values were compared using the Bonferroni test. An oocyte was considered the experimental unit. The residual mean square error term was used to test the fixed effects.

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Exposure of in vitro-matured porcine oocytes to SYBR-14 and fluorescence impairs their developmental capacity.
Exposure of in vitro-matured porcine oocytes to SYBR-14 and fluorescence impairs their developmental capacity

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ABSTRACT

Staining with Hoechst 33342 followed by ultraviolet irradiation is frequently used to aid or confirm the enucleation of recipient oocytes in porcine somatic cell nuclear transfer programs. However, the procedure has a clearly deleterious effect on the developmental ability of oocytes. This study evaluated the effectiveness of a longer-wavelength fluorochrome (SYBR-14) for visualizing maternal chromosomes in in vitro-matured porcine oocytes and the effects of this dye in combination with fluorescence excitation on the subsequent in vitro fertilization and embryo development of the oocytes. In the first experiment, the oocytes were exposed to different concentrations (1, 3, 5 and 7 μg/mL) of SYBR-14 at different incubation times (5, 10 and 30 min) in a 4 × 3 factorial design. The optimal condition for proper metaphase-II plate and first polar body visualization was a 10-min incubation with 5 μg/mL of SYBR-14. In the second experiment, the degeneration rate of the oocytes 18 h after exposure to SYBR-14 (5 μg/mL for 10 min) and fluorescence excitation for 9 or 30 s was significantly higher (p < 0.002) than that obtained for non-exposed oocytes. The fertilization parameters were not influenced by the treatments. The cleavage and blastocyst rates during culture were lower (p < 0.001) for the oocytes exposed to SYBR-14 and fluorescence than for those in the non-exposed group. These results indicate that the exposure of mature oocytes to SYBR-14 and fluorescence for periods as short as 5 s increased the rate of oocyte degeneration and limited their subsequent developmental competence.

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1. Introduction

Somatic cell nuclear transfer (SCNT) is a useful tool for biomedical research. In pigs, the efficiency of SCNT is still very low, although the number of cloned pigs increases each year. The success of the SCNT procedure depends upon numerous factors. One key step is the enucleation of the recipient oocyte. Proper enucleation is essential because it influences the developmental ability of the SCNT embryos. (Peura et al., 1998; Kawakami et al., 2003; Savard et al., 2004). Although several approaches have been described for the enucleation of porcine oocytes, such as “blind” enucleation, squeezing (Lee et al., 2008), centrifugation (Savard et al., 2004; Fahrudin et al., 2007), chemical enucleation (Yin et al., 2002; Li et al., 2009) and Pol-scope microscopy enucleation (Li et al., 2008; Gaamñio et al., 2011), a procedure using bisbenzimidide Hoechst 33342 (H342) staining and ultraviolet (UV) irradiation is frequently used to aid or confirm the enucleation procedure (Polejaeva et al., 2000; Lai et al., 2001; Hyun et al., 2003; Lee et al., 2003, 2008; Kragh et al., 2005; Estrada et al., 2008; Fujimura et al., 2008; Das et al., 2010; Koo et al., 2010; Park et al., 2010; Biswas et al., 2011; Hickey et al., 2011; Terashita et al., 2011). However, it is widely assumed that the exposure of the
recipient oocytes to H342 and UV irradiation might have harmful effects on their DNA and cytoplasmic organelles (Li et al., 2004). We have recently reported that H342 combined with UV irradiation negatively affected the development of in vitro-fertilized porcine oocytes, with the effects being more drastic with increased exposure to UV irradiation (Maside et al., 2011). H342 has a short wavelength (350-nm excitation) and transfers high amounts of energy to the biological material during excitation with possible damaging effects on the developmental potential of the oocytes (Dominko et al., 2000). The use of longer-wavelength fluorochromes should allow lower energy transfer and, therefore, result in less harm to the oocytes. In cattle, Dominko et al. (2000) reported that SYBR-14, a membrane-permeant nucleic acid stain (485-nm excitation), could be employed for enucleation as an alternative fluorochrome to H342 because SYBR-14 did not compromise the developmental potential of the oocytes post-activation. However, to the best of our knowledge, no studies to date have investigated such effects in other species, including pigs. The aim of this study was to investigate the effect of SYBR-14 staining and exposure to fluorescence on the development of porcine oocytes matured in vitro to establish an efficient enucleation method that does not compromise the developmental competence of the oocytes.

2. Materials and methods

2.1. Collection and culture of cumulus-oocyte complexes

The ovaries of pre-pubertal hybrid gilts (age: 5.5–6 months; weight: 90–100 kg) were collected from a local slaughterhouse and transported to the laboratory within 1 h after collection at 35°C in 0.9% NaCl containing 70 µg/mL Kanamycin. The cumulus-oocyte complexes (COCs) were aspirated from medium-sized ovarian follicles (3–6 mm diameter) using an 18-gauge needle connected to a 10-mL disposable syringe. The COCs were washed in a modified Dulbecco’s phosphate-buffered saline (mPBS) medium composed of 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.5 mM CaCl₂·2H₂O and supplemented with 4 mg/mL bovine serum albumin fraction V (BSA; Sigma–Aldrich Quimica SA, Madrid, Spain), 0.3 mM sodium pyruvate, 5 mM D-glucose and 70 µg/mL of Kanamycin. Oocytes with a compact cumulus cell complex and a dark, evenly granulated cytoplasm were washed three times in maturation medium. The maturation medium used was BSA-free North Carolina State University (NCSU-23; Petters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid (FF), 0.8 mM cysteine, 10 ng/mL epidermal growth factor and 5 nm 9-cis retinoic acid. The porcine FF was collected from 3- to 6-mm diameter follicles from ovaries obtained as described above, and centrifuged at 1500 × g for 30 min at 15°C. The supernatant was filtered through 0.8-, 0.45- and 0.22-µm filters, and aliquots were stored at −20°C until use. The COCs were matured in 4-well multidiishes (Nunc, Roskilde, Denmark) containing 70–80 COCs per well in 500 µL of maturation medium supplemented with 10 IU/mL equine chorionic gonadotrophin (Folligon, Intervet International B.V., Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotrophin (Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain) for 20–22 h. The oocytes were then incubated for another 20–22 h in maturation medium without hormones. Maturation was performed under mineral oil at 39°C in a humidified atmosphere of 5% CO₂ in air. After maturation, the COCs were denuded with 0.1% hyaluronidase in the maturation medium by vortexing for 2 min at 1660 rounds/min and were then washed twice in maturation medium. A random subset of the oocytes was mounted on slides and fixed in 30% (v/v) acetic acid in ethanol for 72 h at room temperature. The oocytes were then stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope to assess the meiotic maturation. The oocytes were considered mature when their chromosomes were at metaphase-II (MII) and they had an extruded first polar body (PB1).

2.2. In vitro fertilization and in vitro embryo development

After the treatments, the remaining oocytes were washed three times in pre-equilibrated modified Tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate supplemented with 0.5 mM caffeine and 0.2% BSA (Abeydeera and Day, 1997). The oocytes were then fertilized as described by Gil et al. (2003). Briefly, the oocytes were transferred in groups of 30 oocytes to 50-µL drops of mTBM 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% CO₂ in air for approximately 30 min until the addition of spermatozoa. Semen from a mature Pietrain boar was processed and cryopreserved as described by Carvajal et al. (2004). For each replicate, one pool of semen was made from two straws thawed in a circulating water bath at 37°C for 20 s. After thawing, the sperm were washed three times by centrifugation at 1900 × g for 3 min in mDBPES. Each resulting pellet was resuspended in mTBM, and, after the appropriate dilution, 50 µL of this sperm suspension was added to the fertilization drop containing the oocytes to yield a final concentration of 3 × 10⁵ spermatozoa/mL. The spermatozoa:oocyte ratio was 1000:1.

The gametes were co-incubated at 39°C in a humidified atmosphere of 5% CO₂ and air for 6 h. The presumptive zygotes were removed from the fertilization medium, washed by mechanical pipetting three times in pre-equilibrated embryo culture medium (NCSU-23 with 0.4% BSA) to remove spermatozoa not bound to the zona and transferred into a 4-well multidish (30 zygotes per well), with each well containing 500 µL of the same medium under mineral oil. The presumptive zygotes were cultured at 39°C in a humidified atmosphere of 5% CO₂ in air for 12 h. A randomly proportion of the presumptive zygotes were fixed and stained, as described above, to assess the fertilization parameters. The oocytes with a broken oolemma or abnormal appearance of the cytoplasm were deemed degenerated oocytes. The 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 following fertilization parameters were evaluated: the penetration rate (number of oocytes penetrated/total matured oocytes), monospermy (number of oocytes containing only one male pronucleus/total penetrated), the number of spermatozoa/oocyte (mean number of spermatozoa in penetrated oocytes) and the efficiency of fertilization (number of monospermic oocytes/total matured oocytes).

To examine the ability of the embryos to develop to the blastocyst stage in vitro, the remaining presumptive zygotes were cultured at 39 °C in a humidified atmosphere of 5% CO2 in glucose-free NCSU-23 supplemented with 0.3 mM pyruvate and 4.5 mM lactate for 2 days and then in fresh NCSU-23 medium containing 5.5 mM glucose for an additional 5 days. At 2 and 7 days of culture, the cleavage rate (number of 2- and 4-cell embryos/total cultivated) and blastocyst formation (number of blastocysts/total cultivated and number of blastocysts/total cleaved), respectively, were evaluated under a stereomicroscope. An embryo that had cleaved to the 2-cell stage or beyond was counted as cleaved, and an embryo with a clear blastocoele 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 H342 (Sigma), followed by examination using fluorescence microscopy.

The total number of nuclei that were stained with H342 and displayed blue fluorescence was counted.

2.3. Experimental design

2.3.1. Experiment 1: Visualization of the MI1 plate and the PB1 of in vitro-matured oocytes exposed to different concentrations of SYBR-14 and incubation times

To determine the optimal conditions for enucleation, this experiment was conducted to evaluate the effect of different concentrations of SYBR-14 (Cat. # L7011, Invitrogen, Eugene, OR, USA) and times of incubation on the visualization of the chromosomes at MI1 and the PB1 in the mature oocytes. Immediately after maturation, a total of 913 denuded oocytes from 3 replicates were incubated in 100 µL of maturation medium at different concentrations (1, 3, 5 and 7 µg/mL) of SYBR-14 and incubation times (5, 10 and 30 min) in a 4 × 3 experimental design. Next, the oocytes were washed in maturation medium and individually allocated into drops of 20 µL of maturation medium overlaid with warm mineral oil to be exposed to fluorescence. The MI1 plate and PB1 were visualized by excitation with a fluorescence microscope (Nikon Eclipse TE2000-5®). The fluorescence was observed using a 485-nm excitation line and a 520-nm emission filter. The oocytes were considered correctly stained when both the MI1 and the PB1 were visualized. To confirm the results of visualization, all the oocytes were fixed in acetic acid/ethanol, stained with lacmoid and examined under a phase-contrast microscope, as described above. The efficiency of visualization was defined as the number of oocytes with MI1 and PB1 visualized using live fluorescence evaluation divided by the number of oocytes with MI1 and PB1 visualized using fixation and staining.

The optimal concentration and incubation time considered in this experiment were checked to evaluate the efficiency of enucleation. After 44 h of maturation, 86 denuded oocytes were incubated for 10 min in maturation medium containing 5 µg/mL SYBR-14. The oocytes were loaded individually into 20-µL droplets overlaid with mineral oil. Each oocyte was rotated with the help of two micromanipulators (Eppendorf, Germany) to set the MI1 plate at a 3 o'clock position. The MI1 chromosomes and the PB1 were aspirated using a 17-µm beveled glass pipette. To confirm successful enucleation, all the enucleated oocytes were evaluated by mounting each oocyte on a slide in 4 µL of a dilution of glycerol–DPBS (3:1) containing 10 µg/mL H342, followed by examination using fluorescence microscopy. The efficiency of enucleation was defined as the number of oocytes without residual DNA after enucleation divided by the total number of oocytes subjected to enucleation.

2.3.2. Experiment 2: Effect of SYBR-14 and exposure to fluorescence on the developmental ability of in vitro-matured oocytes exposed to fluorescence

This study was designed to evaluate the effects of SYBR-14 staining and fluorescence excitation for 5 or 30 s on the developmental ability of in vitro-matured oocytes.

The best condition determined from the first experiment was incubation with 5 µg/mL SYBR-14 for 10 min. Immediately after maturation, groups of 30 oocytes were incubated for 10 min in 100 µL of maturation medium containing 0 or 5 µg/mL SYBR-14. Next, the oocytes were washed in maturation medium and individually placed in drops of 20 µL of maturation medium overlaid with warm mineral oil to be exposed or not exposed to fluorescence, according to the experimental design.

A total of 1708 in vitro-matured oocytes from five replicates were randomly allocated into the following groups: Group 1: untreated oocytes (control), Group 2: oocytes exposed to SYBR-14 staining, Group 3: oocytes exposed to SYBR-14 staining and fluorescence for 5 s and Group 4: oocytes exposed to SYBR-14 staining and fluorescence for 30 s.

After the treatments, the oocytes from each group were pooled, exposed to thawed spermatozoa for 6 h and cultured for 12 h (n = 510) to assess the fertilization parameters or cultured for 7 days (n = 1198) to evaluate the embryo development.

In an additional trial conducted as part of this study, the fertilization parameters and the developmental ability of oocytes exposed (5 s or 30 s) or not exposed to fluorescence light were evaluated. We used the same method described above except that the oocytes were not incubated with SYBR-14. A total of 782 in vitro-matured oocytes from three replicates were randomly allocated into the following groups: Group 1: untreated oocytes (control), Group 2: oocytes exposed to fluorescence for 5 s and Group 3: oocytes exposed to fluorescence for 30 s. After treatments, the oocytes were inseminated and cultured for 12 h (n = 280) or for 7 days (n = 502) as described above.
Table 1
Visualization of the metaphase II (MII) plate and the first polar body (PB1) in in vitro-matured oocytes exposed to different concentrations of SYBR-14 for different incubation times.

<table>
<thead>
<tr>
<th>Concentration of SYBR-14 (µg/ml)</th>
<th>Time of incubation (min)</th>
<th>Matured oocytes² (n)</th>
<th>Visualization of MII and PB1</th>
<th>Efficiency of visualization⁸ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Live fluorescence (n)</td>
<td>Fixed-lacmoid evaluation (n)</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>73</td>
<td>0</td>
<td>72</td>
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<td></td>
<td>10</td>
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<td></td>
<td>30</td>
<td>76</td>
<td>60</td>
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</table>

Probability Concentration Time Interaction
0.001
0.001
0.001

Different letters (a, b) denote a significant difference (p < 0.001).

 Only mature oocytes were included in the study.

 The oocytes were considered correctly visualized when both chromosomes at the MII and the PB1 were observed. The efficiency of visualization was defined as the number of oocytes with the MII and PB1 visualized using live fluorescence evaluation/number of oocytes with the MII and PB1 visualized using fixed lacmoid examination.

2.4. Statistical analysis

All data processing and statistical analyses were performed with SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data from all replicates were assessed by analysis of variance using the MIXED procedure, according to a statistical model that included the fixed effects of the SYBR-14 concentrations, incubation times and their interactions (Experiment 1), the treatments (Experiment 2), and the random effect of the replicates in each experiment. Before analysis, the data, expressed as percentages, were modeled according to the binomial model of parameters, as described by Fisz (1980). When the analysis of variance showed a significant effect, the values were compared using the Bonferroni test. The threshold for significance was set at p < 0.05. The results are expressed as the means ± SEM.

3. Results

3.1. Experiment 1

Table 1 summarizes the results of the MII and PB1 visualization in in vitro-matured oocytes exposed to different concentrations and incubation times of SYBR-14. The analysis of variance showed that the dye concentration, the incubation time and the interactions of both factors influenced the percentages of MII and PB1 that could be properly visualized. The efficiency of visualization was highest with a dye concentration of 5 µg/ml and an exposure time of 10 min or longer or with a dye concentration of 7 µg/ml and an exposure time of 5 min or longer. No increase in the intensity of the stain was detected visually after 10 min or with the highest SYBR-14 concentration. To use the minimum concentration of stain required for the visualization of the MII and PB1, 5 µg/ml of SYBR-14 and 10 min of incubation were chosen for the subsequent experiment. In that group, the efficiency of visualization was 81.3 ± 4.9%. In addition, the fluorescence remained observable after 40 min, and 82 out of 86 oocytes were properly enucleated with an efficiency of enucleation of 95.3%.

3.2. Experiment 2

The mean oocyte maturation rate after 44 h of incubation was 80.7 ± 6.1%; the differences among the replicates were not statistically significant. Table 2 shows the degeneration rates for the mature oocytes 18 h after exposure to SYBR-14 (5 µg/ml for 10 min) and fluorescence light for different lengths of time. At 18 h after exposure, the degeneration rates of the oocytes exposed to SYBR-14 and fluorescence were significantly higher (p < 0.002) than those for the other groups.

To evaluate the specific effects of SYBR-14 and fluorescent light on the fertilization parameters, degenerated and immature oocytes were excluded from the analysis, and only mature oocytes were considered. The penetration and monospermia rates, the number of spermatozoa per oocyte penetrated and the final efficiency of fertilization were not influenced by the different treatments (Table 3).

The data on embryo development are presented in Fig. 1. Exposure to only SYBR-14 did not adversely affect the in vitro development of the oocytes to the 2- to 4-cell and blastocyst stages. The cleavage and blastocyst rates were lower (p < 0.001) for the oocytes exposed to the SYBR-14 staining and the fluorescence for 5 s (31.5 ± 2.7% and 7.4 ± 1.5%, respectively) and 30 s (14.9 ± 2.1% and
2.0 ± 0.1%, respectively) than for those in the control group (66.2 ± 2.7% and 27.8 ± 2.6%, respectively). The SYBR-14 and the fluorescence excitation for 5 and 30 s did decrease (p < 0.02) the proportion of cleaved embryos reaching the blastocyst stage in 18.6 and 30.1 points, respectively, compared with the control group. No differences were observed in the blastocyst total cell number among the groups.

Results obtained in the additional trial showed that the exposition of mature oocytes to only fluorescence illumination does not affect the rates of degeneration (range: 0.0–2.3%), fertilization (Table 4) and embryo development (Fig. 2).

4. Discussion

This study was designed to determine whether SYBR-14 may be a possible alternative to Hoechst 33342 to aid or confirm the enucleation procedure in porcine SCNT programs. We evaluated the effectiveness of the SYBR-14 to visualize the maternal chromosomes of mature porcine oocytes, and we studied the effects of this dye in combination with the fluorescence excitation on the subsequent fertilization and embryo development of the oocytes. Because our study was the first study to use SYBR-14 staining in invitro-matured porcine oocytes, a necessary first step was to set up the optimal incubation conditions, dye concentration and exposure time for proper chromosome visualization. Our results from Experiment 1 indicated that the minimal necessary conditions to visualize the MI chromosomes and the PB1 as well as maintain the fluorescence during enucleation were 5 μg/mL of the SYBR-14 and 10 min of incubation. Under these conditions, the efficiency of the chromosome and the PB1 visualization was higher than 80%, and the enucleation was successfully performed in more than 95% of the cases. Furthermore, the enucleation could be accomplished by aspiration of very little cytoplasm surrounding the spindle and the polar body. The minimal concentration of SYBR-14 required for chromosome visualization of porcine mature oocytes was 5-fold higher than that successfully used in bovine mature oocytes (Dominko et al., 2000). The quantitative differences in the requirements of the SYBR-14 between the porcine and bovine oocytes might be due to differences in oocyte cytoplasmic characteristics between the species. For instance, porcine oocytes have larger amounts of lipid distributed throughout the cytoplasm (Genicot et al., 2005).
**Fig. 1.** Effect of SYBR-14 staining and fluorescence excitation for 5 or 30 s on the pre-implantational development of in vitro-matured porcine oocytes. The total number of putative zygotes cultured in each group is given in parentheses. After maturation, the oocytes were incubated for 10 min in maturation medium with 5 μg/ml SYBR-14 and then individually allocated into 20-μL drops of maturation medium overlaid with warm mineral oil to be exposed or not exposed to fluorescence, according to the experimental design. Cleavage rate: percentage of embryos cleaved/total oocytes inseminated; blastocysts/putative zygotes rate: percentage of blastocysts/total oocytes inseminated; blastocyst/cleaved rate: percentage of blastocyst/total of embryos cleaved. The number of cells was evaluated in blastocysts on day 7 of embryo culture (day 0 = day of in vitro fertilization). Bars with different letters are significantly different (at least p < 0.02).

**Fig. 2.** Effect fluorescence illumination for 5 or 30 s on the pre-implantational development of in vitro-matured porcine oocytes. The total number of putative zygotes cultured in each group is given in parentheses. After maturation, the oocytes were incubated for 10 min in maturation medium without SYBR-14 and then individually allocated into 20-μL drops of maturation medium overlaid with warm mineral oil to be exposed or not exposed to fluorescence. Cleavage rate: percentage of embryos cleaved/total oocytes inseminated; blastocysts/putative zygotes rate: percentage of blastocysts/total oocytes inseminated; blastocyst/cleaved rate: percentage of blastocyst/total of embryos cleaved. The number of cells was evaluated in blastocysts on day 7 of embryo culture (day 0 = day of in vitro fertilization).
We used these minimal requirements for the MI and PB1 visualization in the following experiment because the concentration of the stain and/or the length of the exposure to the stain could play an important role in the oocyte developmental capacity.

To our knowledge, this is the first report that evaluates the effects of the SYBR-14 and fluorescence on the developmental competence of in vitro-matured porcine oocytes. Our results denote a clear detrimental effect on the viability of the oocytes and their subsequent developmental ability. The detrimental effects of the SYBR-14/fluorescence were expressed gradually during in vitro culture. The first extensive loss of oocytes occurred 18 h after exposure to SYBR-14 and fluorescence; at this time, more than 20% of the oocytes presented clear signs of degeneration. The deleterious effects tended to be higher (p = 0.1) as the time of fluorescence exposure increased from 5 to 30 s. The penetration and monospermity rates, the number of spermatozoa per oocyte penetrated and the final efficiency of fertilization in the surviving oocytes treated with the SYBR-14/fluorescence were similar to those for the other groups, indicating no effect of the procedure on the mechanisms involved in fertilization. In contrast, the cleavage rates and blastocyst formation strongly decreased in the oocytes exposed to SYBR-14/fluorescence compared with those from the other groups. These percentages decreased (p < 0.05; in the case of the cleavage rate) or tended to be lower (p = 0.09; in the case of the blastocysts rate) as the exposure to the fluorescence increased. Less than 10% of the putative zygotes developed to the blastocyst stage in the SYBR-14/fluorescence groups. The majority of these zygotes stopped development during the first few days of culture and degenerated. Moreover, among the cleaved embryos, only approximately 15% could develop into the blastocyst stage.

All these data together indicate that the exposure of mature oocytes to SYBR-14 and fluorescence presents short- and long-term effects on their developmental ability. A high proportion of the oocytes degenerated 18 h after the treatment, and later embryo development was also profoundly affected in a high percentage of the oocytes. In contrast with our results, Dominko et al. (2000) suggested that SYBR-14 and fluorescence illumination could successfully be used for the enucleation of bovine oocytes without compromising the oocyte developmental competence. However, the results from that work clearly demonstrated that the oocytes labeled with SYBR-14 and exposed to the fluorescence for 30 s had a significantly lower developmental ability than the control oocytes after parthenogenetic activation. This difference could be observed at the time of the first cleavage (82% and 65%) and at the time of the blastocyst formation (37% and 23%) for the control group and for the SYBR-14/fluorescence-exposed oocytes, respectively. Because no oocyte degeneration data are available from that work, we can assume that SYBR-14 and the fluorescence did not induce short-term effects on the bovine oocytes. Furthermore, the long-term effects of the treatment on the bovine oocytes seem to be less drastic than in the porcine oocytes in which the differences in the cleavage rates and blastocyst formation in comparison to the control group were more drastic. It is possible that these species differ in the sensitivity of oocytes to the detrimental effect of the combination of SYBR-14 and fluorescence which could explain these discrepancies.

Our results demonstrated that the treatment of mature oocytes with only SYBR-14 (without subsequent exposure to fluorescence illumination) or with only fluorescence light (without previous SYBR-14 staining) did not interfere with the viability and subsequent developmental ability of the oocyte. The higher proportion of degenerated oocytes and the lower embryo development capacity observed in the oocytes labeled with SYBR-14 and exposed to fluorescence could suggest that SYBR-14 is a photosensitizer that produces injuries to the cells after its incorporation and irradiation. Photosensitization has been previously described for other fluorochromes used in routine analysis techniques, such as Mitotracker Red, which induces apoptosis in intact human cells (Minamikawa et al., 1999). Photosensitization involves the generation of reactive oxygen species (ROS) (Foote, 1968) inside the cell that ultimately undergo apoptosis and cell death (Popovic et al., 1995). In addition, as a consequence of ROS accumulation within the cell, embryonic development could also be impaired. For instance, oxidative damage could cause alterations in the mitochondrial functionality, which have been associated with decreased developmental competence (Van Blerkom et al., 1995; De Matos et al., 1996).

In conclusion, our results demonstrate that the combination of SYBR-14 staining and fluorescence for periods as short as 5 s exerts a clear deleterious effect on the viability and developmental ability of porcine oocytes. These results indicate that SYBR-14 staining should be avoided for enucleation purposes in pigs.

Acknowledgments

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ARTICLE 4

Use of polarized light microscopy in porcine reproductive technologies.
Use of polarized light microscopy in porcine reproductive technologies

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Abstract

The meiotic spindle in the oocyte is composed of microtubules and plays an important role during chromosome alignment and separation at meiosis. Polarized light microscopy (PLM) could be useful for a non-invasive evaluation of the meiotic spindle and may allow removal of nuclear structures without fluorochrome staining and ultraviolet exposure. In this study, PLM was used to assess its potential application in porcine reproductive technologies. The objectives of the present study were to assess the efficiency of PLM to detect microtubule-polymerized protein in in vitro-matured porcine oocytes; to examine its effects on the oocyte developmental competence; to select oocytes based on the presence of the meiotic spindle detected by PLM; and to assess the efficiency oocyte enucleation assisted with PLM. In the first experiment, the presence of microtubule-polymerized protein was assessed and confirmed in oocytes (n = 117) by immunostaining and chromatid detection. In the second experiment, oocytes (n = 160) were exposed or not (controls) to PLM for 10 minutes, and then parthenogenetically activated and cultured in vitro. In the third experiment, development competence of oocytes with a positive or negative signal to PLM was analyzed after in vitro fertilization. Finally, oocytes (n = 54) were enucleated using PLM as a tool to remove the meiotic spindle. A positive PLM signal was detected in 98.2% of the oocytes, which strongly correlated (r = 1; p < 0.0001) with the presence of microtubule-polymerized protein as confirmed by immunostaining. Oocytes exposed to PLM did not differ significantly from controls on cleavage, total blastocyst, expanded blastocyst rates and total cell numbers. The percentage of oocytes at the MII stage and blastocyst formation rate in the negative PLM group significantly differed from control and PLM positive groups. Overall efficiency of spindle removal using the PLM-Oosight system was 92.6%. These results suggest that polarized light microscopy is an efficient system to detect microtubule-polymerized protein in in vitro-matured porcine oocytes and does not exert detrimental effects on porcine oocyte developmental competence. Selecting oocytes by the presence of a PLM signal provides limited improvement on IVF results. Finally, PLM appears as an efficient method to enucleate porcine oocytes.

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Keywords: Polarized light microscopy; Meiotic spindle; Oocytes; Porcine

1. Introduction

The meiotic spindle plays a key role in normal chromosome alignment and segregation during meiosis. The meiotic spindles are assembled by microtubules, as their cytoskeletal components. Methods to
detect and assess the meiotic spindles in oocytes have been described [1]. Compared with methods of spindle evaluation that require oocyte fixation, Polarized Light Microscopy (PLM) offers the unique advantage of being totally non-invasive, preserving oocyte viability and allowing repeated observations of the sample. PLM principles and equipment have been extensively described elsewhere [1–5]. The presence of the meiotic spindle appears to be associated with an increased fertilization rate and embryo quality in mouse and human [1,6–8]. Interestingly, more oocytes with visible spindles fertilized and progressed to blastocysts compared to oocytes without visible spindles [9]. In addition, spindle retardation has been proposed as a marker of oocyte quality in human oocytes and it could be used to select oocytes with an improved embryonic developmental competence [10–12]. The structures and conformation of the meiotic spindles are important to predict the fate of the oocytes. If PLM is able to detect spindle abnormalities, it might become a valuable tool to select oocytes with normal spindle morphology and potentially higher developmental competence.

Within nuclear transfer, PLM can also be used as a tool to assist in the enucleation of oocytes. Several animal species have been successfully cloned by nuclear transfer [13–20]. However, its overall efficiency is still very low [21,22]. Enucleation is an important step towards a successful nuclear transfer and it can be accomplished by different methods such as blind removal of cytoplasm adjacent to the first polar body or by labelling the oocyte DNA with bisbenzimide (Hoechst 33342) and exposure to ultraviolet (UV) light to permit location and removal of chromosomes. Although the latter is a common practice in conventional somatic cell nuclear transfer (SCNT), Hoechst staining and UV light might be detrimental for oocyte developmental competence [23–26].

Research in PLM has been carried out mainly in mice and human oocytes. However, information on the practical use of PLM in oocytes from farm animals, including pigs, is limited and its potential applications in animal reproduction need further assessment. The objectives of the present study were to assess the efficiency of PLM to detect microtubule-polymerized protein in in vitro-matured porcine oocytes; to examine the effects of PLM on the oocyte developmental competence; to select oocytes based on the presence of the meiotic spindle detected by PLM and further assessment of their developmental competence; and to assess the efficiency of PLM as a tool to assist in the removal of the meiotic spindle of metaphase II oocytes.

2. Material and methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich Chemical Company (Alcobendas, Madrid, Spain) unless otherwise indicated.

2.2. Oocyte collection and in vitro maturation

Ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory in saline supplemented with 100 UI/ml potassium penicillin and 100 µg/ml streptomycin sulphate and maintained at 30 °C until oocyte collection. Upon arrival at the laboratory, ovaries were washed three times in sterile 0.9% saline solution. Oocyte collection and maturation were performed as previously described by Gil et al [27] with minor modifications. Briefly, ovarian follicles 3–6 mm in diameter were aspirated with an 18-gauge needle fixed to a 10 ml disposable syringe and cumulus oocyte complexes (COC) containing a compact cumulus mass and even cytoplasmic pigmentation were washed three times in holding medium (TCM199—Invitrogen, Barcelona, Spain—25 mM Hepes and PVA 0.1 mg/ml) and three times in maturation medium. The medium used for oocyte maturation was protein-free TCM199 (Gibco, Life Technologies S.A. Barcelona, Spain) supplemented with 0.57 mM cysteine, 0.1% (w/v) polyvinylalcohol (PVA), 10 ng/ml epidermal growth factor (EGF), 75 mg/ml potassium penicillin G, 50 mg/ml streptomycin sulphate, 0.5 mg/ml LH, and 0.5 mg/ml FSH. The COC (45–50) were transferred into each well of a Nunc (Roskilde, Denmark) 4-well plates containing 500 µl of maturation medium overlaid with mineral oil at 39 °C in humidified air with 5% CO₂. After 22 h in maturation, the oocytes were washed three times in maturation medium without hormonal supplements and cultured for an additional 20 h period. After in vitro maturation, oocytes were denuded of cumulus cells by vortexing with hyaluronidase 0.1 % in holding medium for 2 min. Stripped oocytes were washed three times in holding medium and were randomly assigned to the experimental groups.

2.3. Spindle visualization by Polarized Light Microscopy

Oocytes were placed individually in 10 µl drops of TCM199-Hepes-FCS (TCM199—Invitrogen, Barcelona, Spain, 25 mM Hepes and 10% Fetal Calf Serum) covered with mineral oil in a glass Petri dish. Meiotic spindle visualization was performed using an inverted microscope (Olympus IX71, Japan) at 200x magnification with the Oosight system (CRI, Woburn, MA,
USA) that consists of a liquid-crystal tunable filter optic, a circular polarizer/green interference filter optic, a scientific-grade CCD camera and appropriate software for image acquisition and analysis. During the whole evaluation, oocytes were maintained at 38 °C on a heated stage (ThermoPlate—Tokai Hit, Japan). Each oocyte was rotated using two micromanipulators (Eppendorf, Germany) and micropipettes that help to hold and turn around the oocytes until the meiotic spindle was visualized as a white spot (positive signal) or classified as not having a positive signal.

2.4. Oocyte activation and in vitro culture

Cumulus-free metaphase II oocytes were placed in activation medium (0.3 M mannitol, 1.0 mM CaCl₂ · 2H₂O, 0.1 mM MgCl₂ · 6H₂O, and 0.5 mM Hepes) in a BTX microslide Model #450-1 and were exposed to electrical pulses between 0.5 mm diameter stainless steel electrodes 1 mm apart. Activation was induced with two DC pulses of 1.2 kV/cm for 30 μsec provided by a BTX Electro-Cell Manipulator ECM 2001 (BTX, San Diego, CA, USA). The activated oocytes were washed three times and transferred in groups of 30–40 into each well of a Nunc 4-well dish containing 500 μl of culture medium (North Carolina State University-23 [NCSU-23] medium supplemented with 0.4% BSA) covered with oil. Oocytes were cultured in 5% CO₂ at air in 39 °C for 7 days.

2.5. In vitro fertilization and in vitro culture

In vitro fertilization was performed as described by Gil et al [27]. The basic medium used for IVF was a modified Tris-buffered medium (mTBM), consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and no antibiotics. Briefly, after maturation, oocytes were washed three times with pre-equilibrated IVF medium containing 2 mM caffeine and 0.2% (w/v) BSA (fraction V). After washing, batches of 30 oocytes were placed in 50 μl drops of the same medium covered with warm mineral oil in a 35 mm × 10 mm Petri dish. The dishes were kept in the incubator for about 30 min until spermatozoa were added for fertilization. Frozen semen cryopreserved as described by Roca et al [28], was thawed in a circulating water-bath at 37 °C for 20 s and washed three times by centrifugation at 1900 × g for 3 min in Dulbecco’s PBS (Gibco) supplemented with 0.1%(w/v) BSA, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulphate (pH 7.2). After washing, the sperm pellet was resuspended in IVF medium, and then 50 μl of this sperm suspension was added to the medium that contained oocytes so that each oocyte was exposed to 2000 spermatozoa. Oocytes were co-incubated with spermatozoa at 39 °C in an atmosphere of 5% CO₂ in air for 6 h.

The presumptive zygotes were washed three times and cultured in NCSU-23 medium supplemented with 0.4% BSA at 39 °C in 5% CO₂ in air for 7 d. For assessment of meiotic maturation and fertilization parameters, twelve hours after insemination, a group of oocytes from each experimental group was 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 magnifications of 200x and 400x.

2.6. Microtubule and chromosome immunostaining—confocal microscopy

Oocytes were fixed in 4% (w/v) formaldehyde-PBS, permeabilized using Triton X-100 (2.5% (v/v) in PBS) for 15 min, and simultaneously immunostained for α-tubulin and chromatin detection [29]. Fixation and subsequent incubations were performed at 37 °C. For immunostaining, fixed oocytes were incubated with the anti-α-tubulin monoclonal antibody (mouse IgG1 isotype) (1:4000) for 90 min, followed by incubation with the goat antimouse IgG antibody-Alexa Fluor 488 (Molecular Probes-Invitrogen, UK) (1:500) for 1 h. Between incubations, the oocytes were washed three times in pre-warmed PBS for 5 min. Oocytes were individually mounted on coverslips fitted with a self-adhesive reinforcement ring and then stained with Vectashield – mounting medium for fluorescence with DAPI (1.5 μg/ml) (Vector Laboratories, Inc., Burlingame, CA, USA). The preparation was sealed with nail varnish and protected from light until observation. Negative controls for α-tubulin were produced using only the secondary antibody. A laser-scanning confocal microscope (Leica TCS-SP2-AOBS) was used to examine tubulin (Alexa fluor 488; excitation 488 nm) and chromatin (DAPI; excitation 405 nm). Images were recorded on a computer.

Spindle morphology and chromosomes were classified in three categories: 1) Normal spindle: barrel-shaped with chromosomes clustered as a discrete bundle at the metaphase plate and microtubules crossing the length of the spindle from pole to pole. 2) Abnormal spindle: microtubules were not organized as typical spindles or some microtubules were disassembled with chromosomes with some degree of disarrangement or displacement from the metaphase plate. 3) Absence of
the spindle: no microtubules could be observed around the chromosomes.

2.7. Experimental design

2.7.1. Experiment 1

Oocytes were assessed by polarized light microscopy to detect the presence of microtubule-polymerized protein which could be forming a meiotic spindle. The presence of polymerized protein and a meiotic spindle was confirmed in individual oocytes by immunostaining and chromatid detection. The positive signal detected in each oocyte by the PLM was assessed with the Oosight software to get the retardance value as a putative predictor of microtubule density and conformation. The experiment was replicated seven times.

2.7.2. Experiment 2

Oocytes were exposed or not (controls) to PLM for 10 min. Groups of 10 oocytes were exposed to PLM in 10 µl droplets. Thereafter, the oocytes were parthenogenetically activated and cultured in vitro. Rather than fertilized, oocytes were activated to avoid the use of sperm (male factor) that could affect the IVF outcome. Cleavage rate, total blastocyst rate, expanded blastocyst rate on day 7 and total cell numbers in expanded blastocysts were assessed. Embryo development was assessed as described by Gil et al [27]. The experiment was replicated four times.

2.7.3. Experiment 3

Oocytes were exposed to PLM and assessed to detect the presence or the absence of the meiotic spindle. Control oocytes were not exposed to PLM, so that meiotic spindle was not assessed. Oocytes being PLM positive were handled separately from their PLM negative counterparts. Oocytes from the three experimental groups (control, PLM positive, PLM negative) were fertilized and cultured in vitro as described by Gil et al [27]. Fertilization parameters were assessed in a subgroup of oocytes taken out from each experimental group at 12 h after insemination. The rest of the presumptive zygotes were cultured in vitro for 7 days. Fertilization parameters and embryo development were assessed as described by Gil et al [27]. The experiment was replicated three times.

2.7.4. Experiment 4

For enucleation/meiotic spindle removal, MII oocytes were stained with 1 µg/ml DNA dye Hoechst 33342 and were loaded individually into 20 µl microdroplets. Each oocyte was rotated with the help of two micromanipulators to set the meiotic spindle at 3 o’clock position, followed by aspiration of the second meiotic spindle under polarized light microscopy. Successful removal of the meiotic spindle and chromosomes were confirmed by exposing all the removed cytoplasm to both the PLM-Oosight system and checking for the presence of the removed spindle and to UV light to confirm complete removal of the maternal chromosomes. The experiment was replicated three times.

2.8. Statistical analysis

Data for fertilization parameters, embryo development and retardance values were analyzed by ANOVA using the GLM procedure of SAS [30]. The results are expressed as LSMeans ± SEM. When ANOVA revealed a significant effect, values were compared by Tukey’s test for multiple comparisons. A probability of P < 0.05 was considered to be statistically significant. The correlation analysis was performed using Proc Corr procedure of SAS and the variable analysed were: PLM signal, presence of polymerized protein by immunostaining and spindle morphology by immunostaining.

3. Results

3.1. Experiment 1

A total of 117 oocytes from 7 replicates were assessed. A positive PLM signal was detected in 98.2% of the oocytes (115/117) and 96.5% of them reached the M-II stage (113/117). All of MII oocytes, but one, were positive to PLM. There was a positive correlation (r = 1; P < 0.0001) between the signal obtained by PLM and the presence of microtubule-polymerized protein as confirmed by immunostaining (Fig. 1). A barrel-shape spindle was observed in 92.9% of the MII oocytes by immunostaining (105/113) and an abnormal meiotic spindle conformation was detected in 6.2% of MII oocytes. In addition, the retardance values obtained from in vitro-matured porcine oocytes after being exposed to PLM ranged from 1.87 to 6.38 nm and the mean retardance value of oocytes with normal spindle (barrel shape spindle) (3.9 ± 0.1 nm) did not differ from oocytes with abnormal spindle (3.2 ± 0.4 nm) assessed by immunostaining.

3.2. Experiment 2

A total of 160 MII oocytes for each experimental group from 4 replicates were used in this experiment. After been assessed by PLM, oocytes were pooled together and parthenogenetically activated and cultured in vitro. Oocytes exposed to PLM did not differ significantly from controls in cleavage rate, total blastocyst
Fig. 1. Metaphase II porcine oocyte assessed by (A) conventional light microscopy, (B) polarized light microscopy and (C) after immunostaining-confocal microscopy. (A–B) Original magnification × 200. (C) Original magnification × 154.

rate and expanded blastocyst rate on day 7, respectively (Table 1) (Fig. 2). There were also no differences in total cell numbers counted in expanded blastocysts.

3.3. Experiment 3

In spite of the well known differences between males in IVF results, in this study frozen sperm from a well characterized boar was used to fertilize the oocytes instead of parthenogenetically activating them. The objective was to obtain embryos with the potential to produce live offspring. As shown in Table 2, penetration rates, monospermy fertilization rate and the efficiency of fertilization did not differ significantly among groups. However, the percentage of oocytes that reached the MII stage in the negative PLM group significantly differed from the control and PLM positive groups.

There were no significant differences among groups in cleavage rate. However, significant differences in blastocyst formation rate were observed in the PLM negative group (Table 3). Total cell numbers at blastocyst stage did not differ among control, PLM positive and PLM negative groups.

Table 1
Embryo development of in vitro-matured porcine oocytes after being exposed to PLM and parthenogenetically activated

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cleavage %</th>
<th>% Blastocysts</th>
<th>Total cell counts</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Day 7</td>
<td>XB Day 7</td>
</tr>
<tr>
<td>PLM</td>
<td>160</td>
<td>83.7 ± 1.5</td>
<td>36.9 ± 3.6</td>
<td>21.9 ± 1.7</td>
</tr>
<tr>
<td>Control</td>
<td>160</td>
<td>84.4 ± 1.5</td>
<td>41.2 ± 3.6</td>
<td>26.2 ± 1.7</td>
</tr>
</tbody>
</table>

Day 7, % of total day 7 blastocysts; XB, % of day 7 expanded blastocysts; Total cell counts, Total cell counts in day 7 blastocysts.

3.4. Experiment 4

Fifty four MII oocytes were enucleated under the Oosight system with an overall efficiency of 92.6%. The procedure was simple and a very small amount of cytoplasm was removed in each enucleation (Fig. 3).

4. Discussion

In the present study, PLM was assessed as a tool to apply in porcine reproduction. One of the objectives was to evaluate PLM as an alternative method to detect the meiotic spindle and its conformation. Although darkness attributed to high lipid contents in the cytoplasm of porcine oocytes could hinder the detection of the meiotic spindle, in fact it did not interfere with spindle imaging and a positive PLM signal was detected in 98.2% of matured oocytes. However, effi-

Fig. 2. Embryo development of oocytes exposed to PLM for 10 min and then parthenogenetically activated and cultured in vitro. Original magnification × 100.
Table 2
Effect of oocyte selection by polarized light microscopy on fertilization parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>No. oocytes inseminated</th>
<th>MII oocytes (%)</th>
<th>Oocytes penetrated* (%)</th>
<th>Oocytes monospermic† (%)</th>
<th>Efficiency of fertilization‡ (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>97</td>
<td>96.0 ± 1.6a</td>
<td>72.2 ± 4.8</td>
<td>60.7 ± 6.2</td>
<td>42.4 ± 3.8</td>
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<tr>
<td>PLM-positive</td>
<td>78</td>
<td>100.0 ± 1.6a</td>
<td>66.7 ± 4.8</td>
<td>60.6 ± 6.2</td>
<td>40.1 ± 3.8</td>
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<tr>
<td>PLM-negative</td>
<td>36</td>
<td>80.2 ± 1.6b</td>
<td>70.9 ± 4.8</td>
<td>66.4 ± 6.2</td>
<td>36.5 ± 3.8</td>
</tr>
</tbody>
</table>

Different superscripts within column differ significantly (P < 0.05).

* Percentage of the number of penetrated oocytes/total of MII oocytes.
† Percentage of the number of monospermic oocytes/total of penetrated oocytes.
‡ Percentage of the number of monospermic oocytes/total number of inseminated oocytes.

ciency of detection of the meiotic spindle by PLM is likely to differ according to the quality of oocytes and species specific traits [8,31–35]. In our study, a good correlation between the signal obtained by PLM and microtubule-polymerized protein within in vitro-matured porcine oocytes was observed. Moreover, it was found that 92.9% of the MII oocytes had normal spindle conformation by immunostaining and all of these oocytes, but one, had a positive signal detected by PLM. However, abnormal meiotic spindle conformations were found in 6.2% of the MII oocytes (7/113) and all these oocytes had a positive PLM signal. These results were somewhat discouraging, since PLM was not helpful to distinguish between oocytes with normal or abnormal meiotic spindle conformation. These results, however, are in agreement with reports in human oocytes [34,35]. We agree with Coticchio et al [8] that PLM alone can not provide a detailed description of the MII spindle and the associated chromosomes. Moreover, large segments of highly organized microtubular structures are not an exclusive feature of normally formed spindles, but they are also found in spindles with major morphologic abnormalities or those displaying various degrees of chromosome dispersal [36]. In contrast, other researchers showed evidences that PLM could be a reliable tool to assess the presence of meiotic spindles and their conformation [1,5,37]. All together, we may conclude that PLM is an efficient system to detect microtubule-polymerized protein in in vitro-matured porcine oocytes, but meiotic spindle conformation is unlikely to be reliably assessed by PLM.

Retardance value has also been proposed as a marker of meiotic spindle conformation and also has been suggested as a predictor of oocyte quality in humans and mice [9–12]. Our results do not support this conclusion, since mean retardance value in oocytes with normal meiotic spindle conformation did not differ from oocytes with abnormal spindles, which is consistent with findings from Coticchio and coworkers [36]. Therefore, the proposal that the spindle birefringence could be an indicator of spindle normalcy and chromosome alignment should be carefully considered.

In the present work PLM did not exert detrimental effects on pig oocyte developmental competence. These results agree with those using human [1,32,38], mice [23] and bovine [39] oocytes, that suggest that PLM is a harmless technique that did not compromise either the preimplantation development or the pregnancy rates. We can conclude that PLM is a safe and effective method for imaging meiotic events in living oocytes which does not affect oocyte developmental competence.

If PLM can be used as a tool to select oocytes with improved developmental potential, it will provide a great advantage to some reproductive technologies such as in vitro embryo production, oocyte cryopreservation.

Table 3
Effect of oocyte selection by polarized light microscopy on embryo development

<table>
<thead>
<tr>
<th>Group</th>
<th>No oocytes</th>
<th>Cleavage (%)</th>
<th>Blastocysts (%)</th>
<th>XB (%)</th>
<th>Total cell counts</th>
</tr>
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<tr>
<td>Control</td>
<td>100</td>
<td>70.5 ± 5.7</td>
<td>25.5 ± 1.8a</td>
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<td>PLM-positive</td>
<td>96</td>
<td>62.0 ± 5.7</td>
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<td>PLM-negative</td>
<td>23</td>
<td>53.1 ± 7.6</td>
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<td>1.4 ± 2.2b</td>
<td>19.7 ± 6.1</td>
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Different superscripts within column differ significantly (P < 0.05).

PLM, Polarized Light Microscopy; Blastocysts, % of total day 7 blastocysts; XB, % of day 7 Expanded blastocysts; Total cell counts, Total cell counts in day 7 blastocysts.
and nuclear transfer. In this study, porcine oocytes were selected based on the detection of a positive signal by PLM to perform in vitro fertilization. Fertilization parameters, as well as cleavage and blastocyst formation rates, did not differ between PLM positive and control oocytes. However, PLM negative oocytes showed reduced maturation and blastocyst formation rates. These results are not unexpected since lack of a positive signal could be due to different causes such as incomplete maturation, oocytes with abnormalities (i.e., absence of meiotic spindle or disturbance in tubulin polymerization), and temperature fluctuations during evaluation, which can affect tubulin polymerization/despolymerization. In our experiment the detection of the meiotic spindles were performed after 42–43 h of in vitro maturation, time at which a high percentage of oocytes had reached the MII stage. Thus, it is likely that the results we obtained with PLM negative oocytes were due to the presence of immature oocytes and/or some abnormal oocytes, more than a physiological progression throughout meiosis [40]. Our results agree with reports showing that more human oocytes with visible spindles subjected to intracytoplasmic sperm injection (ICSI), fertilized and progressed to blastocysts as compared to oocytes without visible spindles [7,9,32,38,41]. However, in none of the above referred studies a control group was included in the experimental design as we did in our study. Contrary to the work in human, that uses a limited number of highly valuable oocytes, mostly coming from infertile and/or older women, in our work oocytes were obtained from slaughterhouse ovaries and selected before placing them in maturation. Therefore, the selection pressure exerted on oocytes is probably higher than in works dealing with human oocytes. In addition, assessment of the meiotic spindle when 300–500 oocytes are waiting for evaluation is a time consuming process, which could delay the overall process of in vitro embryo production. Routine application in porcine reproductive biotechnology would require PLM to show a clear advantage and improvement over conventional morphological procedures to select oocytes. At least in our study, such as improvement was limited. However, the use of PLM could be beneficial when individual oocytes become highly valuable (humans) or manipulation of individual oocytes is required (farm animals SCNT, ICSI). In addition, PLM could be a useful resource to study spindle dynamics during physiological events such as maturation and oocyte activation [10,42], and to assess changes in the meiotic spindle during aneu-
gens exposure in dose–response studies and in toxicology experiments [5,37,43–46].

Recently, PLM has been used with promising results to remove the meiotic spindle and chromosomes during nuclear transfer in mice [23], cattle and buffalo [24], and non-human primates [26,47]. The efficiency of such techniques with MII oocytes was very high: 100% in mice [23] and thersus macaque [25,26]), and 88.1% in long-tailed macaque [47]. In our study, we obtained a 92.6% of efficiency and the material removed was confirmed by both PLM and exposure to UV light for complete removal of the meiotic spindle and the chromosomes, respectively. The few failures observed occurred in the first attempts to use the Oosight equipment and could be explained for the lack of training and they were solved with practice. Although more research is needed to assess the advantage of using PLM to improve nuclear transfer, PLM reveals as an effective method to enucleate MII porcine oocytes and a potential alternative to Hoechst staining-UV light exposure.

To the best of our knowledge, this is the first report on the use of PLM in porcine reproduction. More research is needed in related areas such as oocyte cryopreservation before PLM could be implemented with success in porcine reproductive biotechnologies.

In conclusion, our study shows that PLM is an efficient system to detect microtubule-polymerized protein in in vitro-matured porcine oocytes. Images obtained by PLM highly correlate with images obtained by immunostaining of the meiotic spindle. However, PLM itself did not provide a detailed description of the MII spindle conformation and the associated chromosomes. In addition, PLM is not detrimental to porcine oocyte development in vitro, but selection of PLM positive oocytes provides limited improvement on fertilization and blastocyst rates. PLM appears to be an efficient method to enucleate porcine oocytes, susceptible of being used within the whole process of nuclear transfer in pigs.

Acknowledgments

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References


GRAPHIC APPENDIX
FIGURE 1

Collection of cumulus-oocytes complexes.
A and B, ovaries obtained from prepubertal gilts; C, aspiration of follicles to obtain cumulus-oocytes complexes; D, sedimentation of follicular fluid; E, visualization of follicular liquid content under stereomicroscope; F, selection of oocytes with a compact cumulus mass and a evenly granulated cytoplasm.
**GRAPHIC APPENDIX**

**FIGURE 2**

*In vitro maturation.*

A, in vitro fertilization laboratory; B, immature cumulus-oocytes complexes; C, cumulus-oocytes complexes after 20h of *in vitro* maturation; D, cumulus-oocytes complexes after 40h of *in vitro* maturation; E and F, detail of *in vitro* matured-oocytes.

**FIGURE 3**

*In vitro fertilization.*

A, denuded *in vitro* matured-oocyte before *in vitro* fertilization; B, oocytes and spermatozoa bound to the zona pellucida after 6h of *in vitro* fertilization; C, presumptive zygote after 18h of *in vitro* fertilization and 2-cell embryo (arrow).
**FIGURE 4**

Fixation and staining to assess the maturation and the fertilization parameters.

A and B, oocytes or presumptive zygotes mounted on slides with vaseline; C, fixation in a solution of acetic acid: ethanol (1:3); D, staining with Lacmoid in 45% (v/v) acetid acid.
**FIGURE 5**

*Maturation and Fertilization parameters.*

A, immature oocyte at germinal vesicle; B, immature oocyte with metaphase plate (arrow); C and D, *in vitro* matured oocytes with metaphase plate (arrow) and visible first polar body (asterisk); E, monospermic zygote with two pronuclei; F, polyspermic zygote with four pronuclei.
FIGURE 6
Embryo development parameters.
A, in vitro 2- to 4-cell embryos; B, in vitro blastocysts; C, assessment of total number of cells in
in vitro blastocyst with Hoechst 33342 staining and ultraviolet irradiation.

FIGURE 7
Collection and selection of in vivo oocytes and preimplantation embryos.
A, general anesthesia maintained with isofluorane (3.5 - 5%); B, a mid-line incision to expose the
reproductive tract; C, flushing the oviduct and/or the tip of each uterine horn to obtain oocytes
and preimplantation embryos; D, the recovery medium; E, zygotes; F, 2- to 4-cell embryos;
G, blastocysts.
**FIGURE 8**

Exposure to Hoechst 33342 and ultraviolet irradiation.
A, Hoechst 33342; B, in vitro-matured oocytes allocated individually into 20 μL droplets overlaid with mineral oil and exposed to ultraviolet irradiation; C, visualization of an in vitro-matured oocyte exposed to Hoechst 33342 stain and UV irradiation.

**FIGURE 9**

Exposure to SYBR-14 staining and fluorescence.
A, SYBR-14; B, in vitro-matured oocytes allocated individually into 20 μL droplets overlaid with mineral oil and exposed fluorescence; C, in vitro-matured oocyte exposed to SYBR-14 stain and fluorescence.
FIGURE 10

Enucleation of *in vitro*-matured oocytes after SYBR-14 staining.
A, *in vitro*-matured oocyte with the first polar body at 3 o’clock position; B, metaphase plate and the first polar body are visible by exposure to fluorescence after SYBR-14 staining; C, the metaphase plate with small amount of cytoplasm is removed using a 17 μm beveled glasspipette without fluorescence; D, the metaphase plate aspirated using a 17 μm beveled glasspipette under fluorescence.
# APPENDIX

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