UNIVERSIDAD DE CÓRDOBA

DEPARTAMENTO DE GENÉTICA

TESIS DOCTORAL

"Análisis citogenético y celular de la maduración ovocitaria, la capacitación espermática y el desarrollo embrionario temprano en el proceso de la fecundación in vitro en Bos taurus"

Sebastián Demyda Peyrás

Directores:

Prof. Dr. Dr. H.C. Miguel Moreno Millán

Prof. Dr. Enrique Género

Córdoba, 2013

TITULO: *Análisis citogenético y celular de la maduración ovocitaria, la capacitación espermática y el desarrollo embrionario temprano en el proceso de la fecundación in vitro de Bos taurus*

AUTOR: *SEBASTIAN DEMYDA PEYRAS*

© Edita: Servicio de Publicaciones de la Universidad de Córdoba.
Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

D. Miguel Moreno Millán, Profesor Titular de la Universidad de Córdoba, España y **D. Enrique Género,** Profesor Adjunto de la Universidad Nacional de Lomas de Zamora, Argentina

INFORMAN

Que el trabajo titulado "Análisis citogenético y celular de la maduración ovocitaria, la capacitación espermática y el desarrollo embrionario temprano en el proceso de la fecundación *in vitro* en *Bos taurus*", realizado por D. Sebastián Ezequiel Demyda Peyrás, bajo nuestra dirección, se considera finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

Córdoba, a 17 de abril de 2013

Fdo.: D. Miguel Moreno Millán

Fdo.: Enrique Género

TÍTULO DE LA TESIS: Análisis citogenético y celular de la maduración ovocitaria, la capacitación espermática y el desarrollo embrionario temprano en el proceso de la fecundación *in vitro* en *Bos taurus***.**

DOCTORANDO: D. Sebastián Demyda Peyrás

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El doctorando ha llevado a cabo a lo largo del desarrollo de la presente tesis trabajos tanto de revisión bibliográfica, experimentación en laboratorio y análisis de datos como de redacción de las comunicaciones de los resultados. Asimismo, ha completado su formación realizando dos estancias de investigación en dos países europeos (Italia y Bélgica), una de las cuales se extendió por el período de 4 meses, dando cumplimiento a los requisitos para obtener la mención internacional de la tesis.

El desarrollo de la tesis ha sido satisfactorio, dando lugar a diversos trabajos de investigación de los que se han publicado los tres que se adjuntan Además se han originado otros trabajos adicionales también publicados. Todos ellos han sido publicados en revistas internacionales listadas en el primer y segundo cuartil superior de sus respectivas categorías en el "Journal Citation Report" (Thomson Reuters). Todo lo anterior permite la presentación de la presente tesis bajo la modalidad de "Compendio de artículos".

Por todo ello, autorizamos la presentación y defensa de la tesis doctoral.

Córdoba, a 15 de abril de 2013

Fdo.: D. Miguel Moreno Millán

Fdo.: Enrique Género

Esta investigación ha sido financiada por el grupo PAI AGR-158 “MERAGEM” de la Universidad de Córdoba. Sebastián Demyda Peyrás ha obtenido una beca predoctoral MAEC-AECID, dependiente de la Agencia Española de Cooperación Internacional y Desarrollo AECID del gobierno de España durante el período 2009-2012. Las estancias realizadas en el exterior fueron financiadas por el programa de ayudas para estancias a fin de obtener el doctorado con mención europea (2010 – 2011) de la Universidad de Córdoba.

A Alberto, Nora, Julieta y Soledad.
Mi papá, mi mamá y mis dos hermanitas.
NADA de lo poco que soy lo sería sin ellos.

Este trabajo está dedicado a todas las personas con las que me acompañaron durante estos maravillosos años de aventuras por las Europas, y que de una u otra manera me ayudaron no abandonar cuando las cosas iban mal.

A **Miguel Moreno**, que más que un jefe es un amigo. Siempre ahí, para lo que sea. Poniendo la cara miles de veces y peleándose con ciento y la madre para que esto salga adelante. En mi próxima tesis espero que me toque otra vez un jefe como "er Migué".

A **Kike Género**, sin el cual ni estaría acá, ni hubiese durado tanto tiempo por la FCA. Probablemente seguiría ordeñando vacas. Gracias por siempre estar ahí para decir "Tranquilo Seba, no te desesperes".

A **Angelines** (superjefa) y **ANDRÉS** (Va con mayúsculas). Por la paciencia, la ayuda, la oreja, la buena leche, los análisis estadísticos imposibles, las charlas interminables, la paciencia (de nuevo) etc., etc., etc. Bondad y sabiduría concentradas a la millonésima potencia. Demostración clara que el karma es una mentira, habiendo tanta mala gente que sigue jodiendo por este mundo. Una de las cosas que más voy a extrañar de la UCO es poder ir al despacho de la jefa y preguntarle como anda.

A **Antonio Molina,** por los buenos consejos a lo largo del camino por el empuje para ir siempre hacia adelante con cosas nuevas e ideas y proyectos y más cosas nuevas constantemente. Es un placer trabajar con gente buena y capaz a la vez. ☺

A Don **Antonio Rodero,** por las largas charlas en el pasillo, por la humildad que tienen los que son grandes de verdad que debería ser contagiosa y por preguntarme cuanto me falta para terminar la tesis una

vez por mes durante los dos últimos años. Verlo de vez en cuando y charlar un rato le carga las pilas a cualquiera.

To **Ann Van Soom** (the big boss) for gave me the possibility to be in Belgium just by looking my T-shirt. Without doubts, one of the most beautiful and interesting parts of my trip was in the RBU of the Ghent University. It was like to be in in the NASA for almost fourth months. ☺

To the magical team in Ghent. The Spanish and the Belgian ones: **Miguel, Ruben, Alfonso, Hilde, Leen, Josine, Bart, Mohammad, Mohamed, Eline, Karen** and the rest. Especially to my Spanish friends, that introduce me in the fabulous world of Kriek beers and the Borderejte.

To **Isabel Lemahieu** and **Petra Van Damme**, the heart and lungs of the RBU. Thanks for the patience and the guidance. Every lab in the world MUST have a pair of technicians like Petra and Isa. Believe me!!

To "Profesore" **Dino Di Berardino** and their crew **Alfredo** and **Davide** (and later the big **Marcello**), for the beautiful days in Nápoles eating pizzas and trying to make chromosomes glow in the dark!.

A mis amigos y colegas **Manuel Hidalgo (R)** y **Jesús Dorado (B)**. Por suerte las generaciones cambian y vienen profes nuevos que conjugan sabiduría, buena leche y amabilidad. Y para mí, no hay cosa mejor que trabajar con gente buena y sabía. Gracias por los cafés a las 10:15 en la pecera y por tantas horas de corregir los disparates que escribía. Y por los miércoles de feria!. Ylas presentaciones en los cursos que los hacián sudar la gota gorda. Solo espero que el futuro no encuentre haciendo lío juntos de nuevo.

A los niños de Repro: **Dani, Lucre, Isa, Marijó, Maite y Luisa**. Pedazo de grupo que tienen formado por allí. Solo cosas buenas les esperan.

A los moleculares del AGR158, **Alberto** (master yoda), **Mari Carmen, Carmela, Pedro, Loli, Esther, Nacho, Gabriel, Rocío, Rosa, Sara, Marina, Marisol, Jaouad, Mercedes**, etc. Gracias por la paciencia. Por los ratos compartidos. Por enseñarme a usar guantes para cargar las cajas de puntas y correr geles. A **Cristóbal**, por "alegrarnos" el día con su humor exquisito durante 5 añitos. Por los cumples con comida. Por las catas de jamón intempestivas. Por los perolitos y las cenas de fin de año. Y en ellos además agradecer a todos los demás compañeros que me sacaron las castañas del fuego en reiteradísimas ocasiones.

A la **gente buena** de la FCA, UNLZ, la cual me apoyo incondicionalmente por más de tres años, pensando que algo bueno podía salir de mi. Especialmente a **Rosita Galian y Mónica Astiz Gasso**, que confiaron en mí hace mucho tiempo cuando no sabía muy bien que hacer y nadie me daba un lugarcito donde cobijarme.

A la Dra. **Mónica Ritta**. Compañera de desventuras por los sures. Charlas largas y llenas de sabios consejos que nadie más podía dar por allá.

A los amigos de la FCA, que se alegraban mucho de verme de tanto en tanto: **Guillermo, Claudio, Juan Carlos, Ángel, Sergio, Nora, Eduardo, El vasco, Cesar, José María, Fernando**, **Ezequiel, Marcelo, Luis, Alberto, Carolina,** etc., etc., etc.

A los **profes** que tuve a lo largo de todos estos años sintetizados en un solo nombre: **Amado Bozzo.** Clara demostración que se puede ser humilde, sabio y buena gente a la vez cuando uno ama lo que hace.

A **Nando**, por haberme enseñado lo poco que se de vacas.

A **Carlos, Jaouad, Raúl, Tere, Tito, Tania, Sira, Eva** y los demás del camino. Con amigos así no se necesita mucho más.

A **la Dra. Eva Madrid Herrero**, de Madrid Herrero ediciones, que puso en orden todos estos papeles para que parezcan una tesis. Y que además me banco más de la cuenta cuando me daban las malas.

A la gente del grupo de genética vegetal **Carlos, Eva, Raúl, Patri, Mahmoud, Gaby, Latifeh, Teresa, Alfredo, Ángela, Lola** y los demás, por la interminable cantidad de ensaladas compartidas.

A todos los que no nombre todavía (**Profes**, **becarios**, **técnicos**, etc.) del **Departamento de Genética** de la Universidad de Córdoba sintetizados en la persona de **Mari Carmen Mata**. Se agradecen mucho los buenos días y las pequeñas charlas a lo largo de 4 años. La verdad es que me sentí en casa durante todo este tiempo en el bendito C5. Se va a extrañar mucho el ir cantando por los pasillos…☺

A **Córdoba**, con sus Patios, sus Catas, sus Cruces, su Feria, su Noche Blanca, su Mezquita, su Judería con sus callejuelas, su Corredera, el Moriles, etc., etc., etc. Un pedacito mío bien grande se queda por acá.

A la **Universidad de Córdoba**. Un lugar donde la gente te respeta, te aprecia y te ayuda. Un lugar que durante 5 años fue mi casa y al cual voy a extrañar profundamente en el futuro.

A **Josefina**. Esperándome siempre a la vuelta de cada viaje como si no me hubiese ido y preguntándome cuando me iba a quedar ya de una buena vez.

A **Fidelito** y a **Amelie**. A **Bilardo** y **Misha**. Al gateado y al moro. A las terneras, las vaquillonas y las vacas. Y a todos los bichos que me alegraron la vida en todos y cada uno de los momentos que compartieron conmigo.

A **Miguel y Josefa**, que seguro se alegrarían mucho de verme doctor.
A **Fernando** y su hijo **Isidro** (mi sobri). El kukusín que llegó con lo justo para aparecer en esta tesis y cortar la mala racha que llevábamos.

Y finalmente a la persona más importante de todas: **María Gabriela Mattera**. La mejor. Piedra fundamental, brújula, pilar y horizonte durante este viaje. Destino de éxito asegurado. Esta tesis nunca hubiera sido posible sin tener a la Gaby cuidándome.

Han sido 5 años de aventuras, frustraciones, trabajar de sol a sol, cobrar poco, centrifugar cosas, ir a feria, beber más de la cuenta, ir a los congresos, a las cruces, ir al matadero, pinchar ovarios, contar espermatozoides, teñir ovocitos, hablar con la gente, aprender muchísimo, deprimirme porque nada iba bien, estar eufórico porque me aceptaban un paper, comer ensaladas, ir a esquiar, ir a bucear y a la playa, viajar a muchos lados que nunca hubiera pensado, conocer gente y más gente y darme cuenta que ser científico es una vocación para la cual se nace.
Y sin embargo, al final del camino, esto es lo mejor que me ha pasado en mi vida profesional. Y eso es gracias a la **buena leche** de la gente, algo que por otros lados no abunda pero por la Universidad de Córdoba y por los demás destinos en los que estuve durante este viaje hay de sobra. ☺
Gracias a todos ustedes y espero verlos por Argentina o por donde ande muy prontito. ☺

Resumen

La utilización de la fertilización *in vitro* (FIV) en el ganado bovino ha experimentado en los últimos años un gran avance en su implementación a nivel industrial, principalmente en el continente americano. En la actualidad, la FIV se expande a pasos agigantados utilizándose cada día en más animales pertenecientes a distintas razas y distintos países. Si bien es cierto que la eficiencia de la FIV en el bovino está dentro las más altas en los animales de producción, ésta ha sufrido un estancamiento, manteniéndose en los niveles alcanzados desde hace ya varios años. En los últimos tiempos se ha realizado un gran esfuerzo intentando optimizar los protocolos de laboratorio teniendo en cuenta una gran cantidad de factores que, como se ha demostrado, condicionan los resultados obtenidos. Un factor a considerar es sin dudas la raza del animal. En este sentido se han observado diferentes respuestas en las distintas razas bovinas estudiadas. También ha sido detectada una mayor tasa de anomalías cromosómicas en los embriones producidos por FIV, lo cual conspira sin lugar a dudas contra la eficiencia que presenta esta técnica. Es por ello que durante el desarrollo de la presente tesis hemos investigado estos dos efectos descritos mediante una serie de experimentos divididos en tres capítulos.

En el primer capítulo, centrado en la raza bovina de Lidia, hemos demostrado que sus ovocitos se adaptan correctamente a dos protocolos de maduración y fertilización *in vitro* utilizados de manera rutinaria en otras razas. También se ha observado que la cantidad de ovocitos recuperados de cada ovario Lidia es menor que la observada en otras razas estudiadas previamente.

En el segundo capítulo hemos analizado muestras seminales crioconservadas de 4 razas bovinas andaluzas en peligro de extinción (Berrendo en Negro, Berrendo en Colorado, Cárdena Andaluza y Pajuna). Hemos comprobado que estas dosis se adaptan perfectamente a los protocolos estándar de capacitación espermática *in vitro*, lo cual es requisito fundamental para su utilización en programas de FIV. Además este análisis nos ha permitido comprobar que el esperma de las distintas razas se comporta de manera diferencial cuando es capacitado *in vitro* bajo diferentes condiciones de cultivo. Los resultados obtenidos se han visto influenciados por el medio y tiempo de capacitación empleados de manera diferencial en cada una de las razas evaluadas. Esto nos ha permitido inferir que la validez de los test de fertilidad previamente descritos, basados en los resultados de su capacitación *in vitro,* son al menos cuestionables cuando evaluamos animales de distintas razas.

En el tercer capítulo hemos evaluado la aparición de anomalías cromosómicas en los embriones derivados de ovocitos seleccionados de acuerdo a su calidad morfológica y a los protocolos de maduración ovocitaria empleados. En una serie de tres experimentos secuenciales hemos probado que la elección de ovocitos morfológicamente superiores, su maduración durante periodos de tiempos adecuados y en medios de incubación suplementados apropiadamente puede reducir de la aparición de este tipo de aberraciones citogenéticas, mejorando la eficiencia general del proceso de FIV.

Como conclusión hemos demostrado a lo largo de este desarrollo experimental que los protocolos utilizados actualmente en la IVF bovina deberían ser adecuados a cada situación particular teniendo en cuenta numerosos factores, como ser la raza de los animales utilizados y la

posibilidad de reducir las tasas de anomalías cromosómicas para optimizar la eficiencia general del proceso.

Summary

The use of *in vitro* fertilization (IVF) in cattle has experienced in recent years a breakthrough in its commercial implementation, mainly in the Americas, rapidly expanding to animals of different breeds in different countries. The efficiency of IVF in cattle is within the highest in animal production. However, its rates has been maintained at the same point reached some years ago. In recent years, several researchers have been trying to optimize laboratory protocols considering a large number of factors which have been demonstrated conditioning results. One important factor to consider is the breed of the animal used, mostly because there were observed different behaviors in different cattle breeds studied previously. Another cause that has also been detected contributing the lack of efficiency is a higher rate of chromosomal abnormalities in IVF embryos. Due that we focused this thesis to investigate these two effects described through an experimental design dived into three chapters.

In the first one, which focuses on Lidia bovine breed, we have shown that their oocytes can be matured and fertilized using standard laboratory protocols previously used in other breeds. We have also observed that the number of oocytes retrieved from each Lidia breed ovary is lower than that observed previously in other breeds.

In the second chapter we analyzed cryopreserved semen samples from the four most important endangered Andalusian cattle breeds (Berrendo en Negro, Berrendo en Colorado, Cárdena Andaluza and Pajuna). We found that these doses are suited perfectly into standard *in vitro* capacitation protocols, which is a prerequisite for its use in IVF programs. This analysis also allowed us to verify that the sperm of the

diverse breeds has differential behavior when is capacitated *in vitro* under different culture conditions. The results observed have been influenced by the culture media and capacitation time used in a differential way upon the breed evaluated. This allowed us to infer that the validity of previously reported fertility tests, based on the results of *in vitro* capacitation are at least questionable when evaluating animals of different breeds.

In the third chapter we evaluated the occurrence of chromosomal numerical abnormalities in embryos derived from oocytes selected according to their morphological quality and oocyte maturation protocols used. In a series of three sequential experiments we have shown that the use of higher morphologically oocytes, the appropriate maturation period and the adequate supplementation of the maturation medium can drastically reduce the occurrence of this type of cytogenetic aberrations, improving the overall efficiency of the process FIV.

In conclusion, we have determined that the protocols currently used in IVF should be adjusted for each particular situation taking into account, among other factors, the breed of the animals used and the possibility of reducing the occurrence of embryonic chromosomal abnormalities to improve the overall efficiency of the whole IVF process over time.

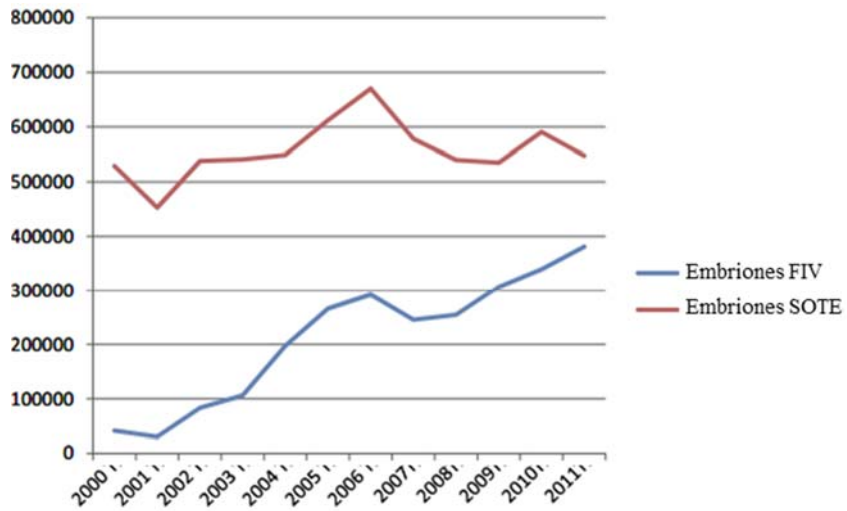
Introducción General/General Introduction

1. Producción de embriones in vitro en el bovino

Desde que en el año 1982 un grupo de investigación en la Universidad de Pensilvania (Brackett *et al.* 1982) lograra el nacimiento del ternero "Virgil", gestado a partir de un embrión bovino obtenido mediante de la fertilización y posterior cultivo *in vitro* de un ovocito preovulatorio se produjo una de las mayores revoluciones en la historia de la reproducción bovina. Este nacimiento fue el primero de los cientos de miles producto de la implementación masiva de la fertilización *in vitro* (FIV) en el ganado vacuno, tercera generación de técnicas de reproducción asistida (ARTs, Assisted Reproductive Techniques) (Thibier 2005). En un principio su desarrollo comercial fue escaso y lento, hasta que en el año 1987 el grupo de investigación de la Universidad de Dublín logró el avance definitivo en la materia con el nacimiento de un embrión derivado de la fertilización de un ovocito inmaduro que fue cultivado *in vitro* hasta desarrollar completamente su capacidad reproductiva (Lu *et al.* 1987). Con el paso del tiempo esta técnica, que en sus comienzos tuvo una tasa de suceso muy baja, fue ganando en eficiencia lo que derivó en su rápida expansión a nivel comercial. Esto fue posible merced al trabajo de muchos grupos de investigación de todo el mundo, que han logrado transformar hoy en día a la FIV en la biotecnología reproductiva más utilizada en la producción comercial bovina.

El desarrollo de la FIV ha sido muy dispar a lo largo del mundo, siendo Sudamérica su principal polo de crecimiento y utilización. Esto se debe, probablemente, al tipo de sistema de producción bovina utilizada en esas regiones basada en rodeos con grandes cantidades de madres que actualmente se haya en un proceso de expansión continua. En ellos, una de las principales necesidades es la de multiplicar exponencialmente la

cantidad de hijos obtenidos de las vacas de mayor mérito genético, logrando de esta forma aumentar la producción obtenida en calidad y cantidad. (Pontes *et al.* 2010). Este desarrollo regional es liderado por Brasil, quien es por lejos el principal productor mundial de embriones fertilizados *in vitro* con el 86% del total, habiendo transferido 325.349 embriones solo en el año 2011. Para tener una idea objetiva del avance y desarrollo reciente que ha tenido la FIV bovina solo hace falta comparar los volúmenes producidos en los últimos años de manera comercial (Figura 1). En Sudamérica el número total de embriones en el año 2002 fue de solo 668, elevándose a 453.471 el año 2012. Esto equivale a un incremento de 678 magnitudes en un período de tan solo 10 años (IETS 1998-2012). En ese mismo período, la producción de embriones *in vivo* mediante superovulación y posterior transferencia embrionaria (SOTE) pasó de 452.546 embriones en el año 2002 a 590.561 embriones en 2011, incrementándose solo el 30,42%. De mantenerse este ritmo de crecimiento las previsiones de la *International Embryo Transfer Society* (IETS) indican que el número de embriones producidos mundialmente por FIV será mayor al número de embriones producidos por SOTE a muy corto plazo (IETS 1998-2012).



Fuente: IETS

Figura 1: Producción de embriones bovinos *in vitro* (FIV) e *in vivo* (SOTE) a nivel mundial durante los últimos 12 años.

2. Usos de la FIV

El empleo de las técnicas de reproducción asistida puede modificar la dinámica de un rodeo animal actuando desde diferentes perspectivas. Sin embargo, es la capacidad de poder multiplicar fácilmente el número de descendientes que podemos obtener de una hembra determinada el mayor impacto que tienen las técnicas de FIV en las poblaciones animales (Rodriguez-Martinez 2012). Esta capacidad es muy superior incluso a la producción de embriones *in vivo* mediante SOTE. Es por ello que es relativamente común su uso con fines diversos como ser la preservación y multiplicación de animales pertenecientes a razas en peligro de extinción (Solti *et al.* 2000) o para la producción de un mayor número de embriones por año en hembras de muy elevado valor genético (Wu and Zan 2012). También se ha utilizado para la multiplicación de animales de

razas relativamente jóvenes compuestas por animales eco-adaptados a ambientes de producción marginales (Gyrolando brasileña (Pontes *et al.* 2010)) y el rescate de animales de alto valor genético que no pueden producir embriones por SOTE o que debieron ser sacrificados debido a enfermedades o traumas (Van Soom *et al.* 1993).

En los últimos tiempos, mayormente en Brasil, se ha comenzado a combinar la producción de embriones FIV con la utilización de técnicas de separación espermática en base a al cromosoma sexual que porte. Esto permite predeterminar con grados de confiabilidad muy altos (>95%) el sexo del embrión de acuerdo a las necesidades productivas: Hembras en el caso de producción lechera o vacas madres o multiplicación de hembras con existencia limitada o bien machos en el caso de que se quisieran producir toros descendiente de una cierta línea materna específica. (de Lima *et al.* 2011). En el mismo sentido, se ha logrado poder obtener una muestra del material genético de embrión en sus primeros estadios, mediante técnicas de biopsia embrionaria, permitiendo el posterior desarrollo del mismo sin sufrir trastorno alguno (Cenariu *et al.* 2012). Esto permite realizar un análisis genómico preimplantacional, descartando a los individuos que presentan anomalías en su genoma o bien favoreciendo el uso de animales que posean caracteres favorables (Humblot *et al.* 2010).

Como bien sabemos, el progreso genético de un grupo de animales depende en gran medida del tiempo trascurrido entre dos generaciones de animales seleccionados. En este sentido el uso de la FIV ha logrado importantes avances al lograr producir embriones a partir de ovocitos de terneras prepúber. Esto ha permitido un importante acortamiento del intervalo generacional al obtener embriones de terneras

hasta 14 meses antes de lo habitual, logrando un incremento sustancial de la mejora genética por unidad de tiempo. (Khatir *et al.* 1998).

Finalmente, en el año 1997 con el nacimiento de "Rosie", la primera vaca transgénica del mundo se produjo el último gran adelanto biotecnológico en la producción de embriones bovinos *in vitro*, dando luz verde a las técnicas reproductivas de quinta generación (Wall *et al.* 1997). Originariamente estos animales fueron concebidos como biorreactores, para ser utilizados en la producción de leche cuya composición se asemejase a la leche humana (Zuelke 1998), o bien en la producción de sustancias específicas utilizadas en el tratamiento de diversas enfermedades como el factor V de coagulación humana, hormona de crecimiento, etc. (Young *et al.* 1998). Con el paso del tiempo estos animales comenzaron a ser modificados con otros fines como ser su utilización como modelos animales para el estudio de enfermedades humanas, e inclusive para la producción de órganos humanos sustitutos, conocidos como xenotrasplantes (Niemann and Kues 2003).

3. Eficiencia de la FIV

La producción *in vitro* de embriones en el bovino es probablemente la más exitosa entre todas las desarrolladas hasta la fecha en las distintas especies de animales domésticos. Todas sus etapas han sido perfeccionadas tecnológicamente hasta niveles razonablemente aceptables desde hace ya varios años. Sin embargo, como en todo proceso realizado *in vitro*, sus niveles de eficiencia aún distan de ser similares a los obtenidos cuando los embriones son producidos por los animales mediante la SOTE (Havlicek *et al.* 2005). Estos niveles de eficiencia contrastan claramente con los observados en otras especies

animales. Es así que en el caballo solo existen dos reportes muy antiguos de potros nacidos a partir de un embrión producido por FIV (Hinrichs 2013). Esto se debe a que aún no se ha logrado hasta la actualidad que el espermatozoide equino pueda fertilizar ovocitos i*n vitro*, principalmente por problemas en la penetración de la zona pelucida. En el perro el problema se encuentra en la fase de maduración ovocitaria y cultivo embrionario, cuyo desarrollo no es el adecuado(Lee *et al.* 2007). Sin embargo, resultados similares se han obtenido en la oveja, la cabra el cerdo (Duszewska *et al.* 2012) y el ratón de laboratorio (Lacham-Kaplan and Trounson 2008).

Desde hace ya varios años se ha producido un estancamiento en la mejora de eficiencia general del proceso de FIV en el bovino (Lonergan 2007). El porcentaje de blastocitos obtenidos por cada ovocito madurado y fecundado sigue siendo superior en los embriones producidos mediante SOTE en comparación con los embriones producidos por FIV, incluso cuando se utilizan los mismos animales como progenitores (Pontes *et al.* 2009). También se ha observado un mayor porcentaje en el número de preñeces logradas y de nacimientos producidos y un menor porcentaje de mortalidad fetal, mortalidad perinatal y de nacimientos de crías con anormalidades morfológicas cando los embriones implantados son producidos mediante SOTE en comparación a los producidos por FIV (Pontes *et al.* 2010; Duszewska *et al.* 2012). En el caso de la combinación de la FIV con el semen sexado estos resultados han sido todavía peores (Rasmussen *et al.* 2013). Esta falta de eficiencia es aún una limitante en la aplicación de la FIV en a nivel industrial, debido a que el costo unitario de cada cría nacida resulta muy elevado en la mayoría de los países. La excepción a este problema es la situación de Brasil, en donde una legislación más adecuada y una producción

ganadera masiva ha permitido la instalación de esta técnica de manera rutinaria (Viana *et al.* 2010b).

Muchas han sido las causas señaladas como responsables de la baja eficiencia de la FIV comparada con la obtenida utilizando SOTE (Lonergan 2007). En la actualidad, las dos principales hipótesis de trabajo son la existencia de condiciones de cultivo embrionario sub-óptimas y la falta de calidad intrínseca de los gametos utilizados debido a la menor selección morfo-fisiológica producida en el laboratorio (Hansen 2006). Estudios recientes demuestran que los sistemas de cultivo *in vitro* no pueden proveer del medioambiente dinámico necesario, que es vital para el desarrollo embrionario en la misma forma que lo hace el útero materno (Hinrichs 2013). Este medio ambiente sub-óptimo se traduce en superiores niveles de stress embrionario, produciendo una merma en su calidad, un descenso en su tasa de crecimiento y una mayor tasa de fracaso (Havlicek *et al.* 2005). Además, la falta de mecanismos apropiados de selección gamética, como los existentes en el tracto femenino, y el uso de métodos masivos para su maduración promueven la puesta en cultivo de gametos que serían eliminados en el útero materno de manera temprana, evitando mayores tasas de fracaso embrionario (Van Soom *et al.* 2007). Es por ello que las investigaciones actuales están enfocadas en determinar cuáles son los períodos críticos en los que se observan las mayores diferencias entre el desarrollo observado utilizando los diversos sistemas de maduración ovocitaria (Albuz *et al.* 2010); de cultivo embrionario (Hyslop *et al.* 2012) y de selección morfológica de los embriones (Sugimura *et al.* 2012), en pos de igualar el nivel de los embriones producidos *in vivo.*

4. El ovocito en la FIV

La maduración ovocitaria en el bovino es un proceso que comienza en la vida fetal con la multiplicación de las células germinales que formarán los folículos primarios. Este proceso queda detenido en la primera división meiótica en el estadio de vesícula germinal (VG) (Eddy *et al.* 1981). Posteriormente y durante el período de foliculogénesis, el desarrollo nuclear del ovocito se reanuda, temporalmente, expulsando su primer corpúsculo polar para quedar detenido nuevamente en su segunda división meiótica en el estado de metafase II durante el momento de la ovulación (Hyttel *et al.* 1997). Durante este período también se desarrolla la maduración citoplasmática. Esta produce una serie de cambios en los orgánulos y en la corona celular que rodea al ovocito preparándolo para poder ser fertilizado correctamente (Carolan *et al.* 1996). Los ovocitos empleados en las técnicas de FIV provienen normalmente de dos orígenes distintos: o bien de ovarios de animales sacrificados por diversas causas, o bien de la punción folicular guiada por ecografía (OPU) (Mapletoft and Hasler 2005). En cualquiera de los dos casos su recuperación se produce deliberadamente mucho antes de momento de la ovulación. Esto se realiza de esta manera principalmente debido a que los folículos preovulatorios contienen ovocitos envejecidos que suelen tener una altísima tasa de fracaso en su desarrollo embrionario (Boni *et al.* 2002). Además, cuando el ciclo estral se halla en su etapa preovulatoria, el folículo dominante induce la atrofia de todos los demás folículos de la onda, lo que redunda también en una menor calidad para el uso en FIV (Viana *et al.* 2004). Es por ello que los ovocitos bovinos normalmente completan su maduración citoplasmática y nuclear mediante un proceso

de cultivo *in vitro,* conocido como MIV (maduración *in vitro*). Desde un principio este procedimiento fue relativamente exitoso con porcentajes de eficiencia cercanos al 40% (Fukui *et al.* 1982), los cuales y merced al desarrollo de la técnica se encuentran hoy rondando el 75-80% (Nagai 2001). Sin embargo, se ha demostrado posteriormente que ciertos factores que no afectaban la tasa de maduración *in vitro* de los ovocitos producían efectos deletéreos importantes el desarrollo embrionario subsecuente (Van Soom and De Kruif 1996). Esto ha sido probado repetidas veces utilizando distintos sistemas de maduración ovocitaria (Russell *et al.* 2006a) e incluso analizando la cantidad de genes relacionados con la viabilidad celular que se expresan diferencialmente en los diferentes sistemas de MIV utilizados (Knijn *et al.* 2002).

4.1. Factores que afectan la maduración ovocitaria *in vitro.*

En los últimos 20 años se ha demostrado la alta sensibilidad que presentan los ovocitos durante su MIV en relación a los resultados obtenidos en los procesos de FIV. Entre los factores analizados la calidad inicial de los ovocitos a madurar juega un papel decisivo. Aquellos que presenten un citoplasma de aspecto obscuro y homogéneo (Fukuda and Enari 1993) y que además se encuentran rodeados por la corona radiata en estado compacto (Blondin and Sirard 1995) serán los ovocitos que tendrán las mejores tasas de desarrollo. Por el contrario, la capacidad potencial de desarrollo de los ovocitos que no cumplan con estos parámetros será muy limitada, e incluso nula en algunos casos.

Los ovocitos bovinos son normalmente madurados durante 20-24 h en medios de cultivo glucosados, que además poseen una serie de nutrientes que permiten su desarrollo. Las condiciones de cultivo

incluyen ambientes húmedos, una baja tensión de O_2 y temperaturas que rondan los 38ºC (Sirard *et al.* 1988). Se ha observado que tanto el tiempo de maduración durante el cual se incuba al ovocito (Birler *et al.* 1998) como la temperatura de incubación empleada (Edwards *et al.* 2005) son también factores que modifican drásticamente el correcto proceso de maduración ovocitaria.

Sin embargo uno de los factores más estudiados durante la MIV es el medio de cultivo utilizado. Actualmente, el TCM-199 (*Tissue Culture Medium 199)* desarrollado en los años 50 por Morgan *et al.* (1955) es el medio de maduración ovocitaria más utilizado en el vacuno. Su composición es relativamente básica debido a que fue formulado originalmente para su utilización en explantes celulares de tejidos animales que poseen necesidades nutritivas y hormonales mucho menos específicas que los ovocitos. Sin embargo su adaptación al proceso de MIV ha sido de lo más exitosa en esta especie. No obstante, se ha observado que una amplia serie de factores relacionados con su composición o suplementación producen importantes efectos en el desarrollo ovocitario. Entre ellos se ha estudiado la osmolaridad (Lim *et al.* 1994), la suplementación del mismo con hormonas hipofisarias (FSH y LH) (Yang *et al.* 1993), el uso de estradiol (Beker *et al.* 2002), el uso de diversos factores de crecimiento (Epidermal Growth Factor o Insulin Growth Factor I) (Sakaguchi *et al.* 2000), y la suplementación del medio con distintos tipos de suero animal (Ocana Quero *et al.* 1994) o Albúmina sérica bovina (BSA) (Ali and Sirard 2002). También ha sido demostrado que el uso de distintas fuentes de substratos energéticos y proteicos tiene un rol fundamental en el desarrollo ovocitario óptimo (Rose-Hellekant *et al.* 1998).

En la actualidad cada laboratorio adecúa su propio protocolo de MIV de acuerdo a sus necesidades y resultados. En la mayoría de ellos se utilizan moléculas totalmente definidas (IGF1 o EGF) o bien con sustancias de extracción de origen animal como ser el suero de origen bovino (fetal, de ternero o de buey) o la BSA. Si bien el uso de las primeras, permite una mayor estandarización del proceso al evitar la problemática derivada de las distintas partidas de los suplementos biológicos (Keskintepe and Brackett 1996), la diferencia observada frente a la utilización de suplementos de extracción aún es apreciable (Palasz *et al.* 2006)

5. El espermatozoide en la FIV

Desde su nacimiento, a partir de las células germinales testiculares, y hasta la culminación de su función reproductora con la liberación de su carga genética dentro del ovocito dando lugar al pronúcleo masculino la célula espermática debe sufrir una serie de transformaciones secuenciales que le permiten adquirir su capacidad reproductiva. Este proceso, llamado capacitación espermatica, descrirto por primera vez por Austin (1952) a mediado de los años 50 consta de dos etápas bien diferenciadas: La etapa preparatoria y la reacción acrosómica (Breitbart 2003). Durante la primera de ellas se producen una serie de cambios en la membrana del espermatozoide, relacionados principalmente la extracción de colesterol, que preparan a la célula para el desarrollo posterior de la reacción acrosómica (Visconti and Kopf 1998). En esta segunda etapa se produce la fusión de las membranas interna y externa del saco acrosomal presentes en la cabeza del espermatozoide, produciendo la liberación de una serie de enzimas hidrolíticas que disuelven parcialmente las

proteínas de la zona pelúcida. Esto, sumado a una hiperactivación mitocondrial, facilitará el pasaje espermático a través de la misma, permitiendo la penetración espermática en el ovocito (Byrd 1981).

Este proceso se desarrolla normalmente durante el pasaje espermático por el tracto reproductivo de la hembra culminando en la periferia del ovocito previo a la fecundación (Coy *et al.* 2008). Durante su camino, las células espermáticas entran en contacto con con una serie de estímulos y sustancias luminales que desencadenan de form natural el comienzo de la reacción acrosómica. Pero a la vez, estas células son expuestas a una serie de procesos selectivos que limitan la llegada masiva de los espermatozoides menos viables al sitio de fecundación. Entre ellos podemos nombrar a la gran distancia que debe recorrer el esperma desde el lugar en el cual es depositado hasta el sitio de fecundación, el pasaje a través del cervix y su criptas y el sistema inmune uterino (Van Soom and De Kruif 1996). Todos estos mecanismos de selección espermática e inducción de la capacitación se hallan ausentes durante la fertilización *in vitro*. En ella las células espermáticas son directamente incubadas en la periferia de los ovocitos, evadiendo todo proceso selectivo que pueda regular su cantidad y estado de capacitación (Van Soom *et al.* 2007). Es por ello que en todo programa de FIV se optimizan las condiciones de fertilización de manera sistemática e individual en cada macho utilizado teniendo en, cuenta entre otros factores, su fertilidad, el lote de esperma a utilizar y las técnicas de selección espermática utilizadas, logrando maximizar los porcentajes de embriones producidos (Katska and Smorag 1996). Esto se debe mayormente a la gran variación observada durante el proceso de crioconservación en la calidad espermática entre los diferentes individuos

y razas afectando, entre otros factores, a la estructura del ADN y fertilidad potencial (Mukhopadhyay *et al.* 2011).

5.1. Capacitación espermática *in vitro*

La mayoría de las sustancias presentes en el tracto uterino que han sido señaladas como responsables del desencadenamiento de la capacitación espermática no se encuentran presentes en los medios de cultivo utilizados durante la FIV. Es por ello que lograr que los espermatozoides adquieran la totalidad de su capacidad reproductiva, se hace necesaria la suplementación exógena del medio de cultivo. Este efecto, originalmente observado en el conejo (Brackett and Oliphant 1975), fue logrado posteriormente en el bovino por el grupo de Dr Parrish de la Universidad de Wisconsin utilizando fluído oviductal autologo (Parrish et al. 1989b). Sin embargo, en trabajos posteriores, este mismo grupo ha conseguido establecer la capacitación espermática *in vitro* utilizando sustancias definidas de facil obtención como ser la heparina (Parrish *et al.* 1988b) o sus derivados (Parrish *et al.* 1989a). Fue posteriormente, y gracias al desarrollo tecnológico de los métodos de detección de la reacción acrosómica que evolucionaron desde el uso inicial de la microscopia óptica hasta las técnicas fluorescentes como el CTC (Ward and Storey 1984) o el FITC (isotiocianato de fluoresceína) asociado a aglutininas derivadas del maní PNA o del guisante (PSA) (Cross and Watson 1994), cuando se pudo comenzar a cuantificar sistemáticamente el efecto de los suplementos utilizados durante la capacitación *in vitro* de una manera más fiable. A partir de esos primeros trabajos mucho se ha profundizado en el estudio de los factores que condicionan/modulan dicha capacitación espermática en el bovino. Hoy

en día es sabido que este proceso está influenciado por el tipo de medio de capacitación (Ijaz and Hunter 1989) y los suplementos que se utilicen como factores capacitantes: heparina (Parrish *et al.* 1988b), cafeína (Singh *et al.* 2009); D-penicillamina (Pavlok 2000); lisofosfolípidos (Wheeler and Seidel Jr 1989); norepinefrina (Way and Killian 2002); progesterona (Lukoseviciute *et al.* 2004); BSA (Pavlok *et al.* 2001); alcohol polivinílico (PVP) (Kato and Nagao 2009) e incluso con la utilización de fluido oviductal (Bergqvist *et al.* 2006). En muchas de estas sustancias se ha demostrado también la existencia de una relación entre la intensidad del efecto producido y la dosis de suplemento utilizada (Fukui *et al.* 1990). También se ha podido observar el efecto sinérgico que se produce mediante la combinación de distintas moléculas (Niwa and Ohgoda 1988) Finalmente se ha observado, en todas las sustancias estudiadas, la aparición de un mayor número de células capacitadas a medida que el tiempo de incubación aumenta. Sin embargo, la velocidad de capacitación no es lineal a través en el tiempo y difiere entre las distintas sustancias estudias (Fukui *et al.* 1990; Gliedt *et al.* 1996).

5.2. Fertilidad *in vitro* del esperma en el bovino

En la década del ochenta, un estudio de Parrish *et al.* (1986) utilizando semen crioconservado puso en evidencia la existencia de una respuesta diferencial en los procesos de FIV de acuerdo al semental utilizado (efecto toro), coincidiendo con las diferencias observadas en la fertilidad de los animales durante la concepción natural. El mismo efecto fue observado recientemente con la utilización de esperma previamente sometido a selección sexual mediante citometría de flujo (semen sexado)

(Barceló-Fimbres *et al.* 2011). Este comportamiento puede ser atribuido, entre otras causas, a diferencias producidas durante el proceso de capacitación *in vitro* del espermatozoide (Birck *et al.* 2010). Sin embargo, dichas diferencias en fertilidad también han sido atribuidas a el tipo de tratamiento al cual fue sometido el esperma, el toro en analizado, e inclusive de la estación climática en la cual fueron tomadas las muestras en las zonas subtropicales (Teixeira *et al.* 2011).

Cada semental que va a ser utilizado en programas de FIV, normalmente debido a sus extraordinarios caracteres productivos, es evaluado profundamente desde el punto de vista reproductivo. Basta pensar el efecto que produciría la utilización de un semental con baja fertilidad, que es en la mayoría de los casos hereditaria, en la fertilización de miles de ovocitos. Es por ello que se ha intentado encontrar una estimación fiable de este parámetro entre diferentes animales durante su desempeño en la FIV (Giritharan *et al.* 2005). El grupo de investigación liderado por la Dra. Ann Van Soom pudo determinar que no sería posible clasificar a los toros en base a su fertilidad *in vitro* evaluando un solo parámetro espermático. En ese estudio de Tanghe *et al.* (2002) se determinar esto evaluando la motilidad, la morfología celular y el porcentaje de células vivas y muertas luego de su descongelado; de su selección y de su utilización en la FIV. Sin embargo, estudios posteriores señalaron que un buen parámetro para medir dicha fertilidad podría ser la velocidad de penetración espermática *in vitro* de ovocitos bovinos en condiciones estándar de FIV (Machatkova *et al.* 2008a). Este método es simplemente una prueba de fertilidad en la que se imita el proceso de FIV, con lo que resulta lento y complicado de realizar. Sin embargo se ha sugerido que el estudio de desarrollo de la reacción acrosómica *in vitro* (RA test) podría ser el parámetro buscado para una correcta estimación

de la fertilidad individual de los toros durante la FIV (Molnarova *et al.* 2006), posteriormente confirmado por Costa *et al.* (2010) utilizando semen de animales *Bos indicus*, Mukhopadhyay *et al.* (2008) en esperma utilizado en IA/ET y Birck *et al.* (2010) durante la I.A. utilizando dosis espermáticas reducidas.

6. Influencias raciales en el esperma, los ovocitos y los embriones producidos *in vitro*.

Los resultados obtenidos en la implementación de la FIV en las diversas razas bovinas existentes en el mundo han sido estudiados ampliamente. Hace más de treinta años Fields *et al.* (1979) demostraron que existe una importante influencia racial en ciertos parámetros seminales de los toros, lo cual fue luego confirmado por Söderquist *et al.* (1996) utilizando distintas razas. Estas diferencias han sido detectadas en los distintos parámetros morfológicos del esperma (Shahani *et al.* 2010), es sus parámetros cinéticos y en los resultados obtenidos en diferentes pruebas laboratoriales (Rodriguez-Martinez and Barth 2007; Menon *et al.* 2011). Esto fue también observado al estudiar el ganado cebuino *(Bos indicus)*, ampliamente utilizado en Brasil (Schmidt-Hebbel *et al.* 2000), y más aún entre la razas pertenecientes a las especies *Bos taurus* y *Bos indicus* (Barros *et al.* 2006).

Los mismos efectos raciales fueron observados en diversos factores relacionados con el gameto femenino. Entre otros se han demostrado diferencias en la respuesta a tratamientos SO y en la calidad y cantidad de ovocitos recuperados de animales pertenecientes a distintas razas (Breuel *et al.* 1991) (Pauciullo *et al.* 2012b). De la misma manera que lo observado en el esperma, estas diferencias fueron mucho mayores

cuando se compararon animales pertenecientes a razas *Bos indicus* (Nunes Dode *et al.* 2000), animales pertenecientes a la especie taurus (Getz *et al.* 2008) y sus cruzamientos (de Armas *et al.* 1994). Estas diferencias fueron observadas la cantidad de ovocitos obtenidos por punción folicular (Viana *et al.* 2010a), en el desarrollo y maduración *in vitro* de los ovocitos obtenidos en el matadero para su uso en FIV (Abraham *et al.* 2012), en la cantidad de folículos mayores a 10mm presentes en el ovario (Domínguez 1995), en la resistencia al stress térmico de los ovocitos (Block *et al.* 2002) y la expresión diferencial de genes relacionados con la viabilidad celular (Eberhardt *et al.* 2009). En razas menores en peligro de extinción, cuya variabilidad genética es normalmente mucho menor, se han observado también diferencias en el número de ovocitos obtenidos de cada ovario (Pauciullo *et al.* 2009; Nicodemo *et al.* 2010).

Similares efectos fueron detectados cuando se evaluó la producción de embriones de manera integral. Embriones producidos mediante SOTE tuvieron diferentes tasas de supervivencia de acuerdo a la raza de animal donante cuando eran producidos en climas cálidos desfavorables (Hernández-Cerón *et al.* 2004). Estudios posteriores sugirieron un marcado efecto paterno en la supervivencia de estos embriones, debiéndose principalmente a la raza del toro utilizado (Barros *et al.* 2006). Sin embargo esto datos no coinciden con los presentados por Eberhardt *et al.* (2009), indicando que el efecto es compartido entre la raza del ovocito y del esperma. Estas diferencias fueron también observadas en la cantidad de embriones obtenidos, en la eficiencia general de la técnica de FIV entre las razas pertenecientes a la especie *Bos indicus* (Gyr y Nelore) y las pertenecientes a la especie *Bos taurus*

(Holstein); y más aún cuando se evaluaron sus cruzamientos F1. (Pontes *et al.* 2010; Viana *et al.* 2010b).

7. Anomalías cromosómicas

La dotación cromosomica normal de un bovino esta dada por un número diploide de cromosomas (2n = 60), divididos en 58 autosómas y un par cromosómico sexual (DiBerardino *et al.* 1990). En un animal normal la forma y tamaño relativo de los pares autosómicos es invariable y determinada. Por el contrario, el par sexual constará de dos crosmosomas equis (BTAX en el bovino) en caso de ser una hembra y un crosmoma equis y un cromosoma Y (BTAY en el bovino) en caso de ser un macho. Sin embargo puede darse el caso de observar alteraciones en la forma o en el número de la dotación cromosómica de un individuo. La variación en la forma de algún cromosoma respecto a las definidas durante su estandarización es considerada una anomalía estructural. Por el contrario, las anomalías numéricas son aquellas en las cuales la morfología de los cromosomas permanece intacta pero la cantidad de los mismos esta alterada.

Se puede dividir a las anomalías estructurales de acuerdo a si hay perdida de material genético, las delecciones; si hay una ganancia de material genético, las duplicaciones, o si hay un reacomodamiento estructural sin pérdida ni ganacia de material genético, como es el caso de las traslocaciones y las inversiones. Por lo general este tipo de anomalías estructurales, mayormente dadas en los autosómas producen defectos fenotípicos muy marcados, provocando en en muchos casos la inviabilidad a nivel embrionario o fetal (Lacadena 1996). Este tipo de anomalías son mucho mas frecuentes en la células animales, llegandose a

observar con una frecuencia promedio de 1 cada 10000 replicaciones celulares. Sin embargo la mayoría de estas células aberrantes no progresan debido a que existen diversos mecanismos celulares que las eliminan (King 2008). Curiosamente en el bovino se da uno de los pocos casos conocidos de anomalias estructurales que se han estabilizado, sin provocar efecto fenotípico alguno. Es el caso de la translocación Robertsoniana rob1/29, descubierta hace cerca de 40 años en la raza Sueca Blanca y Roja por el Dr. Ingemar Gustavsson (Gustavsson *et al.* 1976).

En el caso de las anomalías numéricas podemos encontrar dos tipos de aberraciones bien diferenciadas: Las "somías", que se producen cuando la alteración afecta a uno o mas cromosómas individualmente como ser las trisomías o monosomías) o las "ploidías", en las cuales el juego completo de cromosómas (1n=30 en el bovino) de un individuo se encuentran alterado. En este caso podremos encontrar triploidías (3n=90); tetraploidías (4n=120) o bien haploidías (1n=30). Existen reportes de diversos casos de anomalias de tipo numérico descritas en el bovino (Villagómez *et al.* 2009). Ninguna de ellas, hasta la fecha, han dado cuenta de la existencia de animales haploides o triploides viables. Solo se han reportado un par de casos de animales que poseían mas de una línea celular en su cuerpo (mosaicísmo), habiendo sido diagnosticados como diploides-triploides (Dunn *et al.* 1970; Meinecke *et al.* 2007). Esto se debe al efecto letal que poseen este tipo de complementos cromosómicos durante la fase embrionaria (King 1990), habiendo sido relacionadas a la formacion de "molas" fetales, en el ser humano (Jacobs *et al.* 1982). Sin embargo, su ocurrencia es comunmente detectada a nivel embrionario, siendo asociadas a un desarrollo retardado y posterior muerte en una fase muy temprana (Kawarsky *et al.* 1996).

Las anómalias cromosómicas fueron clasificadas según su origen por McFadden and Friedman (1997) como anomalías de tipo primarias, que son aquellas que afectan a la gran mayoría de las células del individuo desde su nacimiento, o bien de tipo secundario, cuando por alguna razón la carga cromosómica de un grupo de células varía a lo largo de su vida de manera caótica. Las anomalías primarias se pueden producir durante la gametogenesis, tanto femenina como masculina,dotando al cigoto, y por ende a todo el indivíduo de una carga cromosómica anormal, o bien en las etapas primordiales de la vida embrionaria, merced a lo cual podremos tener mas de una población celular con distinta carga cromosómica en un mismo individuo (Lacadena 1996).

7.1. Detección de las anomalías cromosómicas

Las anomalías cromosómicas pudieron ser detectadas originalmente mediante la utilización de técnicas citogenéticas convencionales basadas en la microscopia óptica (Murray *et al.* 1985). Posteriormente, con el desarrollo de la hibridación fluorescente *in situ* (FISH) en la Universidad de Cambridge (Telenius *et al.* 1992) se ha logrado mejorar notablemente la capacidad de detección de las mismas permitiendo cuantificar de manera más precisa y real la ocurrencia de este fenómeno pero a un costo mucho más elevado. En la actualidad, este tipo de anomalías se pueden detectar fácilmente mediante el uso combinado de la FISH los marcadores moleculares de tipo STR (Short Tandem Repeats) de manera más rápida y económica (Demyda Peyrás *et al.* 2013). Recientemente, se ha comenzado a implementar el análisis de hibridación genómica comparativa (aCGH) utilizando microarrays. Estas técnicas permiten

incluso detectar, a un costo elevadísimo todavía, pequeños desbalances en los cromosomas que escapan totalmente a la capacidad de diagnóstico de las otras técnicas disponibles de manera rutinaria (Wells and Levy 2003). Este tipo de técnicas genómicas están comenzando a ser utilizadas en embriones animales como lo demuestra el reciente trabajo del laboratorio del Dr. Jiri Rubes realizado en el cerdo (Hornak *et al.* 2012).

7.2. Alteraciones cromosómicas en los ovocitos

El gameto femenino es muy sensible diversos factores que incrementan la aparición de anomalías cromosómicas durante su maduración, que luego se ven reflejadas en la ploidía embrionaria. Esta ampliamente demostrado que factores propios de la hembra favorecen la aparición de anomalías cromosómicas es sus ovocitos. La edad materna o el envejecimiento ovocitario por razones exógenas son sin duda los de mayor preponderancia. Esto ha sido ampliamente demostrado en animales de laboratorio (Liu and Keefe 2002; Takahashi *et al.* 2010), cerdos (Hornak *et al.* 2011) y en los seres humanos (Nagaoka *et al.* 2012). Estas anomalías han sido también correlacionadas positivamente con una baja calidad morfológica inicial del ovocito (Gianaroli *et al.* 2007; Munné *et al.* 2007) y negativamente con el diámetro folicular (Ocana-Quero *et al.* 1999b). Muy poco se ha estudiado acerca de este factor intrínseco de calidad ovocitaria en el bovino. La mayoría de ellos, llevado a cabo por el grupo de Dr Di Berardino en Nápoles, evalúan las tasas de anomalías cromosómicas intrínsecas en ovocitos recuperados de animales fértiles (Nicodemo *et al.* 2010; Pauciullo *et al.* 2012a).

Se ha podido determinar la existencia de una menor tasa de anomalías cromosómicas cuando los ovocitos son madurados *in vivo* en

comparación con los madurados *in vitro* (Xu *et al.* 2008). Asimismo, en ovocitos MIV se ha detectado la influencia del tratamiento de los ovocitos y de los medios de cultivo (A'Arabi *et al.* 1997) y la suplementación de los mismos durante su maduración (Ocana-Quero *et al.* 1999b).

7.3. Alteraciones cromosómicas en el esperma

Los espermatozoides de los animales sufren durante su proceso de maduración una división reduccional que los transforma en células haploides (1n=30 en el bovino), recreando en condiciones normales junto al gameto femenino el número cromosómico diploide de la especie (2n=60 en el bovino). Esto da lugar a la formación y desarrollo del cigoto y posteriormente, a un embrión cromosómicamente normal. Sin embargo está ampliamente demostrada la existencia de espermatozoides portadores de anomalías cromosómicas intrínsecas que, en el momento de la fertilización, producen embriones aberrantes destinados en un altísimo porcentaje al fracaso (Zenzes and Casper 1992).

Las anormalidades en las células espermáticas son normalmente muy bajas en los individuos fértiles (Harton and Tempest 2012), estando correlacionadas estrechamente a individuos con ciertos problemas de fertilidad de tipo morfológico (*teratozoospermia).* Estas aberraciones cromosómicas no se han observado en cambio en individuos con problemas de motilidad o concentración espermática (Ghoraeian *et al.* 2013). Comúnmente, estas aneuploidías espermáticas suelen presentarse como células espermáticas que presentan disomías o nulisomías en el par sexual (Moosani *et al.* 1995). Por otro lado, se ha observado que las anomalías cromosómicas presentes en el cariotipo de un individuo se

trasladan de manera directa a sus espermatozoides, produciendo un efecto hereditario (Vegetti *et al.* 2000). Notablemente, y a diferencia de lo que ocurre en los gametos femeninos, la presencia de estas anormalidades espermáticas se mantiene constante a lo largo de toda la vida fértil del individuo, siendo extremadamente difícil que aumente con el paso del tiempo (Wyrobek *et al.* 2006) en condiciones normales.

En el bovino prácticamente no se han realizado estudios sobre la aparición de aneuploidías en el esperma. En este sentido, el grupo del Dr. Di Berardino ha podido determinar los niveles basales de células espermáticas con anomalías cromosómicas en animales fértiles pertenecientes a diferentes razas vacunas, situándolos en torno al 0,5 – 1% (Nicodemo *et al.* 2009; Pauciullo *et al.* 2009). Posteriormente un estudio realizado sobre 47 toros fértiles situó este nivel más bajo aún, en torno al 0,3% (Rybar *et al.* 2010). Este estudio determinó que, si bien las diferencias observadas en los porcentajes de anomalías espermáticas entre los diferentes toros analizados son mínimas, estas podrían ser suficientes para modular la fertilidad *in vivo* de dichos sementales.

No conocemos hasta la fecha estudios realizados en el vacuno que relacionen la aparición de anomalías cromosómicas en embriones producidos por FIV con la tasa de anomalías cromosómicas presentes en el esperma. Por el contrario, existen varios estudios, con resultados desalentadores, que evalúan el uso de esperma de animales portadores de diversas translocaciones robertsonianas en su cariotipo como sementales en programas experimentales producción de embriones mediante SOTE (Wilson 1991) o FIV (Machatkova *et al.* 2005). De la misma manera no existen estudios que evalúen la tasa de anomalías cromosómicas espermáticas en toros con parámetros morfológicos anormales, debido a que normalmente los mismos son descartados como reproductores.

7.4. Alteraciones cromosómicas en los embriones

Una causa importante de mortalidad embrionaria, que redunda en la baja eficiencia global del proceso de FIV, es la aparición conformaciones cromosómicas numéricas anormales en los embriones (Kawarsky *et al.* 1996). Este fenómeno fue descrito inicialmente en los animales de laboratorio por Tarkowsky (1966) y posteriormente en el vacuno por MacFeely and Rajakoski (1968). Si bien en un principio se sugirió que su prevalencia era baja, esta idea fue modificada gracias al aumento de la eficiencia de detección de este tipo de anormalidades, comprobándose que su ocurrencia era mucho mayor de los que se pensaba (Viuff *et al.* 2002). Está demostrado que este fenómeno se observa en una mucha mayor proporción en los embriones producidos por FIV que en los producidos dentro mediante SOTE (Viuff *et al.* 2001). Incluso se ha observado que el solo hecho de madurar los ovocitos *in vivo* dentro del folículo materno previo a la FIV produce una merma significante en el porcentaje de embriones con aberraciones cromosómicas (Dieleman *et al.* 2002). Esto podría deberse que durante el proceso de FIV confluyen una gran cantidad de factores, que a diferencia de lo que ocurre en el tracto reproductivo femenino, no pueden ser controlados en su totalidad. De acuerdo a numerosos estudios realizados, la aparición de anomalías cromosómicas en los embriones producidos *in vitro* podría depender de la calidad intrínseca de los gametos masculinos (Rybar *et al.* 2010), femeninos (Lechniak and Switonski 1998), las técnicas de reproducción asistida utilizadas (Li *et al.* 2005) y los protocolos utilizados en cada una de esas técnicas (Viuff *et al.* 2000; Lonergan *et al.* 2004).

Es bien sabido que estas anomalías determinan una altísima tasa de fracaso en el desarrollo embrionario, estando relacionadas a pérdidas

durante la etapa embrionaria, fetal. e incluso a anormalidades fenotípicas muy marcadas en las crías resultantes (King 1990). Es por ello que el estudio de esta problemática durante la producción de embriones *in vitro* y la búsqueda de soluciones para limitar su aparición permitirá un aumento de la eficiencia global de las técnicas de reproducción asistida, más importantes día a día en la producción y desarrollo del ganado vacuno (Moreno Millán and Demyda Peyrás 2010).

Objetivos/Objetives

Objetivos

El objetivo general de esta tesis doctoral fue el evaluar diversos factores intrínsecos y extrínsecos que afectan el desarrollo de los gametos y los embriones bovinos durante su maduración, fertilización y cultivo *in vitro* desde un punto de vista fisiológico y citogenético. Este objetivo general se concreta en los siguientes objetivos específicos:

1) Caracterizar las tasas de recuperación ovocitaria a partir de ovarios de vacas de Lidia.

2) Evaluar el efecto de dos protocolos estándar de maduración ovocitaria en las tasas de maduración y fertilización obtenidas *in vitro* en ovocitos provenientes de vacas de Lidia.

3) Evaluar protocolos estándar de capacitación espermática *in vitro* en dosis seminales criopreservadas pertenecientes a razas de bovinos Andaluces en peligro de extinción como condición previa para su uso potencial en programas de fertilización *in vitro*.

4) Investigar el efecto de la raza, la suplementación del medio de capacitación y el tiempo de incubación en los resultados obtenidos en la capacitación espermática *in vitro*.

5) Evaluar el efecto producido por la calidad morfológica intrínseca del ovocitos en la incidencia de la tasa de anomalías cromosómicas en embriones bovinos preimplantacionales producidos *in vitro.*

6) Evaluar la influencia producida por el tiempo de maduración ovocitaria en la incidencia de la tasa de anomalías

cromosómicas en embriones bovinos preimplantacionales producidos *in vitro*.

7) Evaluar el efecto producido por la suplementación sérica del medio de maduración ovocitaria en la incidencia de la tasa de anomalías cromosómicas en embriones bovinos preimplantacionales producidos *in vitro.*

Objectives

The overall objective of this thesis was to evaluate several intrinsic and extrinsic factors that affect the development of bovine gametes and embryos during maturation, fertilization and *in vitro* culture from a physiological and cytogenetic point of view.

1) To characterize the oocyte recovery rates from Lidia cow ovaries.
2) To evaluate the effect of two standard oocyte maturation protocols on *in vitro* maturation and fertilization rates of oocytes derived from Lidia cows.
3) To evaluate standard AR protocols in cryopreserved semen doses of Andalusian endangered cattle breeds, as a condition for its potential use in FIV programs.
4) To investigate the effect of breed, culture medium supplementation and incubation time on AR test results.
5) To evaluate the effect produced by the oocyte morphological quality on the incidence of numerical chromosomal abnormalities in early IVP bovine embryos.
6) To evaluate the effect produced by the oocyte maturation time on the incidence of numerical chromosomal abnormalities in early IVP bovine embryos
7) To evaluate the effect produced by the oocyte maturation media (serum supplementation) on the incidence of numerical chromosomal abnormalities in early IVP bovine embryos,

Capítulo I / Chapter I

"*In vitro* oocyte maturation and fertilization rates in Spanish the Lidia bovine breed"

Demyda Peyrás, S., Hidalgo, M., Dorado, J., Anter, J., Género, E. & Moreno Millán, M. 2013. **Short communication: *In vitro* oocyte maturation and fertilization rates in Spanish the Lidia bovine breed.** Spanish Journal of Agricultural Sciences, *In Press.*

Abstract

The Lidia bovine breed is the most successful cattle bred on the Iberian Peninsula, also considered a hallmark of Spanish tradition and image around the world. However, due to their production system, inbreeding and loss of genetic variability have been described as important issues in certain lineages, causing reproductive problems within herds. To handle these genetic impairments, modern Assisted Reproductive Techniques (ART´s) such as *in vitro* fertilization could be used as an important tool to improve the reproductive efficiency of these animals. To our knowledge, no previous studies have been performed to evaluate the developmental capacity of *in vitro* matured oocytes derived from Lidia cows. Consequently, the aims of the study were to characterize the oocyte recovery rates from Lidia cow ovaries and to evaluate the effect of two standard *in vitro* maturation protocols on oocyte maturation (cumulus expansion and nuclear maturation) and fertilization rates in oocytes derived from Lidia cows after *in vitro* fertilization. For this purpose, 261 ovaries from Lidia cows were processed obtaining 1,125 viable cumulus oocyte complexes (COCs). The oocyte recovery rate obtained (4.31 viable COCs per ovary) was lower than those described previously in other studied breeds. Maturation rates were evaluated in two different oocyte maturation media with (M1) and without (M2) hormonal supplementation. The percentage of COCs with expanded cumulus cells was significant lower in M1 (74.35%) compared with M2 (82.25%). MII rates (67.75% in M1 and 73.18% in M2) were similar to previous studies in different cattle populations. The use of hormonal supplementation in the maturation media (M2) significantly improved the percentage of COCs with their cumulus cells expanded ($p < 0.01$) and

nuclear maturation rates ($p < 0.05$), but it did not affect the fertilization percentages obtained in this experiment. In conclusion, our study suggests that oocytes of the Lidia cattle breed can be obtained, matured and fertilized following standard protocols previously used in other cattle breeds.

Keywords: Lidia breed, Oocyte Maturation, *In vitro* fertilization (FIV)

Introduction

The Lidia bovine breed, also known as fighting the bull is the most successful cattle bred on the Iberian peninsula. It is also considered a hallmark of Spanish tradition and image around the world (Jimenez *et al.* 2007). This animal population is reared following traditional procedures, characterized by a long history of isolation from the rest of Spanish cattle breeds. For hundreds of years, this breed has only been selected for their temperament and aggressiveness without considering other phenotypic characteristics. This fact has caused a loss in genetic variability and an increase in inbreeding depression due to this phenotypic selection performed over the years (Cañón *et al.* 2008). The same authors have determined that this mainly occurs on isolated lineages, where only a few superior animals are the highest contributors to the gene pool (Cortés *et al.* 2011). Bulls of this breed are isolated from the females to increase aggressiveness and are normally killed during a bullfight before being able to produce any offspring (Jimenez *et al.* 2007). Only a very limited number of bulls are pardoned and subsequently used in for reproduction/breeding according to their performance during the Lidia. For this reason, the use of assisted reproductive techniques (ART´s) could be considered as an important tool to obtain more offspring from certain maternal lineages or from a particular bull killed during the Lidia (Katska-Ksiazkiewicz *et al.* 2006). As part of the *in vitro* procedures, artificial oocyte maturation has a significant role in *in vitro* technologies in cattle (Russell *et al.* 2006b). Previous reports suggested that animal breed (Kafi *et al.* 2002; Nicodemo *et al.* 2010; Pauciullo *et al.* 2012a), maturation media (Choi *et al.* 2001) and protocols (Hawk and Wall 1994) had an influence in the developmental capacity of the oocytes matured *in*

vitro. Furthermore, it has been demonstrated that oocyte maturation affects the FIV outcomes in several species and breeds (Palma and Sinowatz 2004). To our knowledge, no previous studies have been performed to evaluate the developmental capacity of *in vitro* matured oocytes derived from Lidia cows. This is mainly due to the difficulty in obtaining a high number of ovaries from this breed to carry out these studies. Therefore, the aims of the study were to: 1) characterize the oocyte recovery rates from Lidia cow ovaries; and 2) evaluate the effect of two standard oocyte maturation protocols on *in vitro* maturation and fertilization rates of oocytes derived from Lidia cows.

Material and Methods

For this purpose, 261 ovaries from 3 to 8 years-old Lidia cows belonging to three different lineages were collected in two replicates at the local slaughterhouse and transported to the laboratory within the 2 h in a 0.9% NaCl aqueous solution at 30-37ºC. Thereafter, the ovaries were washed in a warm physiological saline solution supplemented with kanamycin (25 mg mL^{-1}) until all remaining traces of blood were removed. Cumulus oocyte complexes (COCs) were aspirated from follicles between 4 and 8 mm in diameter with a 18G needle. After sedimentation, oocytes were poured into Petri dishes and selected under a stereomicroscope with a warm plate. Recovered oocytes were classified according to their morphology (Hazeleger *et al.* 1995). Only those with a homogeneous cytoplasm and at least three layers of cumulus cells were used in this study. A series of standard Tyrode´s Albumin Lactate Pyruvate media were used throughout the entire experiment. COCs were washed twice in warm (38.5ºC) TALP media supplemented with hepes salts (H-TALP,

according to Parrish *et al.* (1988b)) and matured in groups of 100 on four well Nunclon^{TM} dishes for 24 h, at 38.5ºC in a 5% CO_2 humid atmosphere. Oocyte recovery rates were recorded for further analysis. To evaluate the effect of the hormonal supplementation on maturation and fertilization rates, oocytes were cultured in two different maturation media: Medium 1 (M1) was TCM199 modified bicarbonate-buffered (Sigma Aldrich Spain), supplemented with 10% of fetal calf serum, 0.4 mmolL^{-1} glutamine; 0.2 mmol L^{-1} sodium pyruvate and 50 mg mL^{-1} gentamicin without any hormonal supplementation; and Medium 2 (M2) was the same media supplemented with 25 μg mL^{-1} FSH, 6.25 μg mL^{-1} LH and 2 μg mL^{-1} estradiol. After maturation, percentages of oocytes with expanded cumulus cells were determined in each group following our laboratory criteria (Ocana Quero *et al.* 1994). A total of 727 cultured oocytes (369 in M1 and 358 in M2) were denuded by vortexing, fixed and stained with standard Hoechst 33342 protocol (Flaherty *et al.* 1995) to evaluate their nuclear maturation status. The remaining oocytes were fertilized with a pool of frozen semen from three different Retinta bulls of proven fertility. For this purpose, thawed spermatozoa were previously selected through a discontinuous Percoll gradient (45 and 90% (v/v); Pharmacia) according to Parrish *et al.* (1995) and adjusted to a final concentration of $1x10^6$ sperm mL^{-1} in equilibrated IVF-TALP medium (Parrish *et al.* 1988b) supplemented with 6 mg mL^{-1} BSA and 20 mg mL^{-1} heparin. Oocytes were washed twice and co-incubated in groups of 60 with sperm at 38.5ºC in 5% CO2 on equilibrated IVF-TALP medium. After 20 h presumptive zygotes were denuded by vortexing and stained as previously described by Flaherty *et al.* (1995), to determinate pronuclei formation rates. Statistical analysis used was a Z-score test (z) with two tails (Demyda-Peyras *et al.* 2012). The intragroup differences

for total viable oocytes, maturation rates and fertilization rates between replicates were achieved using Fisher exact test, using Minitab software Version 15.1(Minitab, Inc, College State, Pennsylvania).

Results and Discussion

Oocyte recovery rates observed in our study are shown in Table 1. A total of 1,356 oocytes of different categories were recovered from 261 ovaries; with an average of 5.20 oocytes per ovary punctured. After morphological selection, 1,125 oocytes were used throughout this experiment, resulting in 4.31 viable oocytes suitable for maturation per ovary punctured. These results were lower than previous reports by several authors in other breeds: 4.60 in Podolian and 5.83 in Maremmana (Pauciullo *et al.* 2012a); 5.33 in Czech Simmental and 6.50 in dairy Holstein (Machatkova *et al.* 2008b); 9.5 in Belgium Holstein and 11.1 in Belgium Blue (Van Soom *et al.* 1993). We only found a lower rate reported previously in Czech beef breeds (Machatkova *et al.* 2008b) and in zebu Nelore cows (Dode *et al.* 2001).

It is noteworthy that the low number of replicates could influence the results obtained. This is due to the great difficulty in obtaining enough ovaries derived from Lidia cows to perform an experiment. However, there were not statistical differences between replicates (Fisher exact test, $p > 0.05$). In this sense, the low number of oocytes obtained per ovary is consistent with the moderate fertility previously observed in this breed (Jimenez *et al.* 2007). Recently, Evans *et al.* (2010) suggested that a lower number of follicles are reflective of the environment during fetal development. It was observed in beef heifers restricted to 0.6 of their maintenance energy requirement, from shortly before conception to

the end of the first trimester of pregnancy. Conversely, Lidia breed cows are reared in extensive production systems in the Spanish "*dehesas"* with no nutritional imbalances throughout the whole year (Jimenez *et al.* 2007). Several authors identify an antagonistic association between high milk production and *in vivo* (Olsen *et al.* 2011) and *in vitro* (Khatib *et al.* 2010) fertility traits. More recently, other authors (Peñagaricano and Khatib 2012) were more specific, suggesting the same association between milk protein yield and *in vitro* fertility. But Lidia breed is characterized by a medium milk production, with no genetic selection performed in this sense. One possible explanation is that oocyte recovery rates can be influenced by their selection process for the last five centuries, focused mainly on their aggressiveness (Silva *et al.* 2006). In this sense, a previous study showed that hostile animals have lower reproductive performance (Phocas *et al.* 2006). Moreover, it has been suggested that the main cause of this lower reproductive performance is the greater basal concentrations of glucocorticoids and catecholamine's shown in more temperamental cattle, leading to a "stress like" situation (Burdick *et al.* 2011). Likewise, high genetic selection pressure only for a few specific production traits might have the same deleterious effect on reproductive traits, as it has been demonstrated in high-producing dairy cows (Walsh *et al.* 2011). Oppositely, effectiveness of selection for reproductive traits has been widely demonstrated (Alvarez *et al.* 2005; Cushman *et al.* 2005). Moreover, recent work has established that the number of COC´s obtained from individual cows in an IVP program can be increased by genetic selection (Merton *et al.* 2009). Finally, another possibility is that the low number of COCs obtained could be derived directly from the animals breed. Recent work reports that the outcome of IVP bovine embryos depends on the breed of the donor ovary (Abraham

et al. 2012). The same differences have also been observed in a native Hungarian pig breed (Egerszegi *et al.* 2001).

Table 1. Oocyte collection rates in Lidia cattle breed

Total animals slaughtered	153
Total ovaries collected	261
Total oocytes collected	1,356
Total oocytes collected per ovary[1]	5.19
Total viable oocytes	1,125
Total viable oocytes per ovary[1]	4.31

[1]Statistical differences between the two replicates were assessed using Fisher exact test. No differences were observed between replicates (p>0.05).

Therefore, morphological quality of the oocytes collected in this study can be also influenced by genetic and breed factors (Domínguez 1995). However, the percentage of viable oocytes obtained in our study was similar to those observed in other breeds (Fischer *et al.* 2000; Ribeiro *et al.* 2011).

Oocyte *in vitro* maturation rates achieved in our study using two different maturation media are shown in Table 2. Highly significant statistical differences ($p < 0.01$) were observed in the percentage of COC´s with expanded cumulus after maturation between M1 (74.35%) and M2 (82.25%). Cumulus cell expansion was higher when the maturation medium was supplemented with FSH, LH and estradiol, as previously demonstrated (Younis *et al.* 1989; Rose and Bavister 1992). Similar results were obtained in nuclear maturation rates: 67.75% in M1 and 73.18% in M2; however, the amplitude differences were statistically

lower than those observed previously in cumulus cell expansion rates ($p < 0.05$). It has been shown that oocyte donor breed affects its developmental competence in other species (Ptak *et al.* 2003; Rátky *et al.* 2005). However, our results were within the average rates previously reported in other cattle breeds (Camargo *et al.* 1997b; Kafi *et al.* 2002; Wang *et al.* 2007; McLaughlin and Telfer 2010; Nicodemo *et al.* 2010). It may suggest that donor breed may not produce an important influence in the *in vitro* oocyte nuclear maturation in some bovine populations.

Table 2. Maturation and fertilization rates of oocytes derived from Lidia breed cows after in vitro maturation in two different maturation media.

	Medium 1		**Medium 2**	
	n	**%**	**n**	**%**
Total oocytes	573	100	552	100
Oocytes with expanded cumulus[1]	426	74.35^{A}	454	82.25^{B}
Oocytes with nuclear maturation[1]	250/369	67.75^{a}	262/358	73.18^{b}
Fertilization rates	98/204	48.03	105/194	54.12

[1]On each row, values followed by different letters (a, b) differ statistically ($p<0.05$), and values followed by different capital letters (A,B) highly differ statistically ($p<0.01$) (two tails Z test for proportions). Statistical differences between the two replicates were assessed using Fisher exact test. No differences were observed between replicates in cumulus expansion, nuclear maturation or fertilization rates ($p>0.05$)

Finally, fertilization rates obtained are in agreement with previous observations by our group (Ocana Quero *et al.* 1995) and with those of other authors (Sumantri *et al.* 1997). However, higher pronuclear formation rates were obtained in previous experiments (Kafi *et al.* 2002). It is noteworthy that fertilization rates were similar in both maturation media, supplemented with hormones or not. This finding is in accordance with previous results obtained by other authors (Sartori *et al.* 2010). Until

now fertilization failures have not been related with oocyte sources in other species or breeds (Squires 2005; England and Russo 2006; Burns *et al.* 2010). However, some recent studies have suggested that the existence of specific genes activated during oocyte maturation play a major role in the fertilization process (Zheng and Dean 2007; Meczekalski 2009). On the other hand, male influence has also been suggested as the primary cause of failed fertilization in livestock (Bar-Anan *et al.* 1980), due to a lack of ability of sperm to penetrate the oocyte (Sartori *et al.* 2010). Despite the controversy found in literature, the acceptable fertilization rates observed in our study do not appear to be an important issue during the *in vitro* fertilization process of Lidia breed oocytes.

Conclusions

Our study suggests that oocytes belonging to Lidia cattle breed can be obtained, matured and fertilized following standard protocols previously described in other cattle populations. However, the total number of COC´s and viable oocytes obtained from ovaries derived from Lidia cows are lower than those obtained in other breeds previously studied. Finally, the use of appropriate hormone supplementation in the maturation media enhances maturation rates, without affecting the fertilization process of these oocytes. Further studies are necessary to optimize the overall success of FIV protocols in this particular breed.

Acknowledgements

Sebastian Demyda Peyrás is supported by a grant from MAEC-AECID (Spain) to perform his PhD thesis. The authors thank COVAP

slaughterhouse for their help in collecting the ovaries and to the Retinto Breeders Association for the semen samples donated. This study was funded by the Animal and Molecular Cytogenetic Laboratory and the MERAGEM (AGR-158) group of the University of Cordoba.

Capítulo II / Chapter II

"*In vitro* induction of the acrosome reaction in spermatozoa from endangered Spanish bulls: effect of breed, culture media and incubation time"

Demyda-Peyrás, S., Dorado, J., Hidalgo, M., De Luca, L., Muñoz-Serrano, A. & Moreno-Millán, M. 2012. ***In vitro*** **induction of the acrosome reaction in spermatozoa from endangered Spanish bulls: Effect of breed, culture media and incubation time.** Livestock Science, 149, 275-281.

Abstract

Current conservation programs for endangered species have been widely benefited from the use of modern assisted reproductive techniques. However, at the present time, there is little information available regarding the use of locally adapted cattle breeds on FIV programs, and even less about their sperm behavior. The aims of this study were to: 1) evaluate standard acrosome reaction (AR) protocols in cryopreserved semen doses of Andalusian endangered cattle breeds, and 2) investigate the effect of breed, culture medium supplementation and incubation time on AR test results. To this end, 80 frozen semen doses from 16 bulls (5 semen samples per bull) of five different cattle breeds (3 Berrendo en Colorado, 6 Berrendo en Negro, 2 Cardena Andaluza, 1 Pajuna and 4 Retinta) were *in vitro* incubated in three test media (Heparin, BSA or their combination), using Sp-TALP as a control, to induce *in vitro* AR. The percentage of acrosome-reacted spermatozoa was evaluated using fluorescence techniques (FITC-PNA and Propidium Iodide) at four time-points (0, 30, 60 or 120 min.). Throughout the whole experiment, spermatozoa incubated with a combination of both supplements (Heparin and BSA) showed significantly ($P<0.05$) higher results than those processed with Heparin or BSA alone. In contrast, the control media (without supplementation) showed the lowest results. We also found a significant ($P<0.05$) influence of cattle breed on the dynamics of the *in vitro* induced AR using different protocols. Based on our results, we can conclude that the response pattern of *in vitro* induced AR depends not only on culture media and incubation time but also on the cattle breed. Therefore, all these factors must be taken in consideration to assess the rate of AR sperm in bulls.

Keywords: Cattle breed, Acrosome reaction, Cryopreserved sperm, Fluorescent techniques.

Introduction

Extensive cattle production in the Spanish region of Andalusia is based on autochthonous breeds well adapted to grazing conditions (Rodero Serrano and Valera Córdoba 2008). There is ample evidence that livestock breeds and populations evolved over the years in stressful environments have a range of unique adaptive traits (e.g. disease resistance, physiological and behavioral adaptations) which enable them to be productive in these environments (Mirkena *et al.* 2010). Limitations on these breeds usually are both the low animal number available for reproductive purposes and the high level of inbreeding existing in their herds. Current conservation programs for endangered species have been widely benefited from the use of modern assisted reproductive techniques, such as the *in vitro* embryo technology (FIV) (Solti *et al.* 2000; Andrabi and Maxwell 2007). However, at the present time, there is little information available regarding the use of locally adapted cattle breeds on FIV programs and even less about their sperm behavior (Teixeira *et al.* 2011).

Recently, Hoflack *et al.* (2006) has recommended a routine semen evaluation consisting of semen volume, sperm concentration, motility and morphology prior to include semen donor bulls into an assisted reproduction program. However, it has been demonstrated that bulls with good sperm motility parameters can have a reduced fertilization capacity due to a failure of the acrosome reaction of sperm (Lessard *et al.* 2011). It may suggest that the classical semen parameters might not be enough to assess the fertilizing capacity of a semen sample. Previous studies have suggested the use of *in vitro* acrosome reaction (AR) test as a valuable tool to predict both field fertility (Waberski *et al.* 1999; Birck *et*

al. 2010) and *in vitro* fertilizing capacity of semen in cattle (Molnarova *et al.* 2006; Costa *et al.* 2010).

Those procedures to induce AR are date back a long time (Parrish *et al.* 1988a). Likewise, heparin and bovine serum albumin (BSA) have been widely used as AR inducers, mainly due to their neutral effect on the oocyte and sperm vitality (Parrish *et al.* 1999). Interacting with these compounds, the incubation time also has an important role in AR tests (Pavlok *et al.* 2001). In fact, it has been widely demonstrated that the percentage of acrosome-reacted spermatozoa increases during the incubation time (Parrish *et al.* 1988a; Hochi *et al.* 1996). However, to our knowledge, there are no studies on the acrosome reaction-inducing activity of either heparin, BSA or their combination in Spanish endangered cattle breeds.

The aims of this study were therefore to: 1) evaluate standard AR protocols in cryopreserved semen doses of Andalusian endangered cattle breeds, as a condition for its potential use in FIV programs, and 2) investigate the effect of breed, culture medium supplementation and incubation time on AR test results.

Materials and Methods

Semen samples

In total, 80 frozen semen samples from 16 bulls (5 semen samples per bull) of five Andalusian endangered native breeds: 3 Berrendo en Colorado (BC), 6 Berrendo en Negro (BC), 2 Cardena Andaluza (CA), 1 Pajuna (PA) and 4 Retinta (RE) were analyzed in this study. Semen collection and freezing was performed at the Animal Reproduction,

Selection and Conservation Center - CENSYRA, (Badajoz, Spain), as described by Januskauskas *et al.* (1996). Only samples with acceptable post-thawing viability and motility (> 40% viable sperm and > 50% progressively motile sperm) were used in this experiment (Birck *et al.* 2010).

Sperm selection

Frozen semen doses were thawed in a water bath at 37ºC for 1 min. Viable spermatozoa were selected by "Swim Up" method as described previously by Shamsuddin *et al.* (1993). Briefly, aliquots of 500µl of semen were carefully placed in the bottom of a 2 ml tube containing 1ml of equilibrated Sp-TALP medium (Sp-T, Caisson Laboratories, USA) and incubated for 1 h at 39ºC, in a 5% CO_2 atmosphere. After incubation, 1ml upper fraction was collected and mixed with 5ml of equilibrated Sp-T in a conical tube and centrifuged (150 x g, 10 min. at 24ºC). The supernatant was discarded and the sperm pellet was resuspended in 3ml of Sp-T and centrifuged again (150 x g, 10 min. at 24ºC). Thereafter, the sperm pellet was transferred to a clean conical tube containing Sp-T medium.

Acrosome reaction status

Acrosome integrity was assessed as described by Cheng *et al.* (1996), with minor modifications. In brief, aliquots (50µl) of semen samples were diluted in PBS to a final concentration of 25 x 10^6 spermatozoa/ml. Droplets (10µl) of the sperm sample smeared onto a microscopic slide were air dried, fixed, and permeabilised with 70% (v:v) ethanol for 30

sec. A mixture of 10µl of propidium iodide (0.1mg/mL) and 20µl of isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (0.1mg/mL) was then spread over each smear. Slides were incubated in a dark and moist chamber at 4°C for 30 min. They were subsequently rinsed with excess deionized water at 4ºC mounted with 10µl of VECTASHIELD® Mounting Media (Vector Laboratories Ltd., Peterborough, United Kingdom), covered with a coverslip, and sealed with nail polish. A total of 100 spermatozoa were counted per slide under 400 X magnification with an epifluorescence microscopy (Reichert-Jung Polyvar, Austria) using a BP 460 to 490 nm excitation filter. Two sperm subpopulations were identified: 1) spermatozoa displaying intensively green fluorescence of the acrosomal cap indicated an intact outer acrosomal membrane, and 2) spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap indicated the process of vesiculation and breakdown of the acrosomal membrane, or spermatozoa displaying a fluorescent band at the equatorial segment or no fluorescence indicated residues or a complete loss of the outer acrosomal membrane, respectively. Values were expressed as percentages.

Experimental design

Eighty frozen semen samples from 16 bulls (5 semen samples per bull) of five different endangered breeds (3 BC, 6 BN, 2 CA, 1 PA and 4 RE) were used in this study. Swim up selected spermatozoa were incubated at 38ºC in a 5% CO_2 atmosphere, in four different culture media: Sp-TALP medium (Sp-T; Caisson Laboratories, USA), Sp-TALP medium supplemented with 0.6% BSA (Sigma Aldrich, Spain, A3311) (Sp-TB), Sp-TALP medium supplemented with 10µg/ml of Heparin (Sigma

Aldrich, Spain, H3149) (Sp-TH), and Sp-TALP medium supplemented with 0.6% BSA and 10μg/ml of Heparin (Sp-TBH). Finally, aliquots were collected at 0, 30, 60 and 120 min. and stained for AR evaluation. Five replications were made.

Statistical analysis

A three-fixed factor design was performed: *animal breed* (BC, BN, CA, PA and RE), *culture media* (Sp-T, Sp-TB, Sp-TH and Sp-TBH) and *incubation time* (T0, T30, T60 and T120). Data were tested for normal distribution using the Kolmogorov-Smirnov test prior to ANOVA, and analyzed using the General Lineal Model (GLM) procedure of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). Individual factors and breed-culture medium, breed-time and time-culture medium interactions were also evaluated, taking into account the imbalance between the number of bulls among breeds. Duncan's Multiple Range procedure was performed to test differences among means. Data were significant at $P<0.05$.

For each culture media and breed combination, the orthogonal contrast test was performed in order to analyze the interaction between the main effect and the interaction effects of the AR status over time.

Results

Significant ($P<0.01$) effects of breed, culture medium and incubation time on AR-status were found. Moreover, significant ($P<0.01$) interactions among all these factors were found. For this reason, each

independent variable (breed, culture medium or incubation time) were then analyzed based on the other two.

Effect of the breed on the in vitro induced AR

As shown in Table 1, *in vitro*-induced AR in bull sperm in response to different culture media and incubation time varied significantly ($P<0.01$) depending on the breed. The *in vitro* AR-induced by Sp-TH was consistent over time only in Retinta bulls (high mean AR values at T30, T60 and T120). For all the other breeds, a random pattern was observed for *in vitro*-induced AR.

Effect of the culture media on the in vitro induced AR

The percentage of induced AR for each culture medium is shown in Table 2. The AR status induced by each culture medium depends on both the breed and the incubation time, with exception of Sp-TBH. This culture medium showed the higher number of reacted sperm cells instead of breed and incubation time (Table 2). At T30 and T120, AR status was significantly ($P<0.05$) higher in samples processed with Sp-TH compared with control medium (Sp-T) and Sp-TB, except for Pajuna bulls at T120. However, at T60, there was no significant ($P>0.05$) differences between Sp-TH and Sp-TB for BC, BN and CA.

Effect of the incubation time on the in vitro induced AR

A positive trend was observed between AR status and incubation time regardless of the breed or the culture medium (Fig. 1). A significant

(P<0.01) linear relationship between *in vitro* induced AR in all breeds and culture media was shown by the orthogonal contrast analysis. However, some of these breed-media combinations (Sp-TB for BC, BN, CA and RE; Sp-TH for CA; and Sp-TBH for CA) were also fitted to a quadratic model.

Table 1: Effect of the cattle breed on the in vitro induced AR test in bull spermatozoa

		Culture Media			
IT	Breed	Sp-T	Sp-TB	Sp-TH	Sp-TBH
T30	BC	7,07±0,34 ^{B}	7,53±0,35 ^{A}	11,33±0,44 ^{BC}	14,33±0,30 ^{B}
	BN	5,13±0,20 ^{A}	9,00±0,29 ^{B}	12,40±0,28 ^{C}	13,97±0,24 ^{B}
	CA	4,80±0,29 ^{A}	6,40±0,37 ^{A}	8,10±0,28 ^{A}	12,40±0,37 ^{A}
	PA	5,80±0,73 ^{A}	7,60±0,68 ^{A}	10,40±0,51 ^{B}	15,40±0,51 ^{B}
	RE	7,53±0,41 ^{B}	9,73±0,43 ^{B}	11,60±0,35 ^{BC}	17,80±0,74 ^{C}
T60	BC	8,20±0,20 ^{C}	13,80±0,38 ^{AB}	14,00±0,45 ^{A}	19,73±0,45 ^{A}
	BN	6,67±0,19 ^{AB}	14,50±0,32 ^{AB}	14,63±0,29 ^{AB}	22,93±0,34 ^{B}
	CA	5,90±0,28 ^{A}	13,20±0,68 ^{A}	13,80±0,59 ^{A}	22,00±0,56 ^{B}
	PA	8,40±0,40 ^{C}	19,20±0,37 ^{C}	17,00±0,45 ^{C}	28,00±0,84 ^{C}
	RE	7,07±0,33 ^{B}	14,73±0,28 ^{B}	15,93±0,37 ^{BC}	23,13±0,48 ^{B}
T120	BC	9,07±0,28 ^{A}	18,00±0,44 ^{A}	20,73±0,37 ^{A}	27,47±0,38 ^{A}
	BN	11,30±0,29 ^{B}	20,33±0,22 ^{B}	23,30±0,32 ^{B}	32,73±0,39 ^{B}
	CA	8,70±0,30 ^{A}	20,60±0,45 ^{B}	22,80±0,42 ^{B}	28,10±0,35 ^{A}
	PA	10,40±0,51 ^{B}	22,20±0,37 ^{C}	20,40±0,68 ^{A}	38,20±1,02 ^{D}
	RE	9,20±0,34 ^{A}	20,40±0,40 ^{B}	21,87±0,43 ^{AB}	36,27±0,64 ^{C}

Culture Media (CM): Sp-T: Sp-TALP; Sp-TB: Sp-TALP + BSA; Sp-TH: Sp-TALP + Heparin and Sp-TBH: Sp-TALP + BSA + Heparin.

Incubation Time (IT): T30: 30m; T60: 60m and T120: 120m. Breed: BC: Berrendo en Colorado; BN: Berrendo en Negro; CA: Cardena Andaluza; PA: Pajuna and RE:Retinta. Values indicate the percentage of live acrosome-reacted spermatozoa expressed as means ± S.E.On each block, values with different letters (A,B,C and D) are significantly different (P<0.05, Duncan's test).

Table 2. Effect of the culture media on the in vitro induced AR in bull spermatozoa

		Cattle breed				
IT	CM	BC	BN	CA	PA	RE
T30	Sp-T	7,07±0,34 ^{A}	5,13±0,20 ±	4,80±0,29 ^{A}	5,80±0,73 ^{A}	7,53±0,41 ^{A}
	Sp-TB	7,53±0,35 ^{A}	9,00±0,29 ±	6,40±0,37 ^{B}	7,60±0,68 ^{A}	9,73±0,43 ^{B}
	Sp-TH	11,33±0,44 ^{B}	12,40±0,28 ±	8,10±0,28 ^{C}	10,40±0,51 ^{B}	11,60±0,35 ^{C}
	Sp-TBH	14,33±0,30 ^{C}	13,97±0,24 ±	12,40±0,37 ^{D}	15,40±0,51 ^{C}	17,80±0,74 ^{D}
T60	Sp-T	8,20±0,20 ^{A}	6,67±0,19 ±	5,90±0,28 ^{A}	8,40±0,40 ^{A}	7,07±0,33 ^{A}
	Sp-TB	13,80±0,38 ^{B}	14,50±0,32 ±	13,20±0,68 ^{B}	19,20±0,37 ^{C}	14,73±0,28 ^{B}
	Sp-TH	14,00±0,45 ^{B}	14,63±0,29 ±	13,80±0,59 ^{B}	17,00±0,45 ^{B}	15,93±0,37 ^{C}
	Sp-TBH	19,73±0,45 ^{C}	22,93±0,34 ±	22,00±0,56 ^{C}	28,00±0,84 ^{D}	23,13±0,48 ^{D}
T120	Sp-T	9,07±0,28 ^{A}	11,30±0,29 ±	8,70±0,30 ^{A}	10,40±0,51 ^{A}	9,20±0,34 ^{A}
	Sp-TB	18,00±0,44 ^{B}	20,33±0,22 ±	20,60±0,45 ^{B}	20,40±0,37 ^{B}	20,40±0,40 ^{B}
	Sp-TH	20,73±0,37 ^{C}	23,30±0,32 ±	22,80±0,42 ^{C}	22,20±0,68 ^{B}	21,87±0,64 ^{C}
	Sp-TBH	27,47±0,38 ^{D}	32,73±0,39 ±	28,10±0,35 ^{D}	38,20±1,02 ^{C}	36,27±0,43 ^{D}

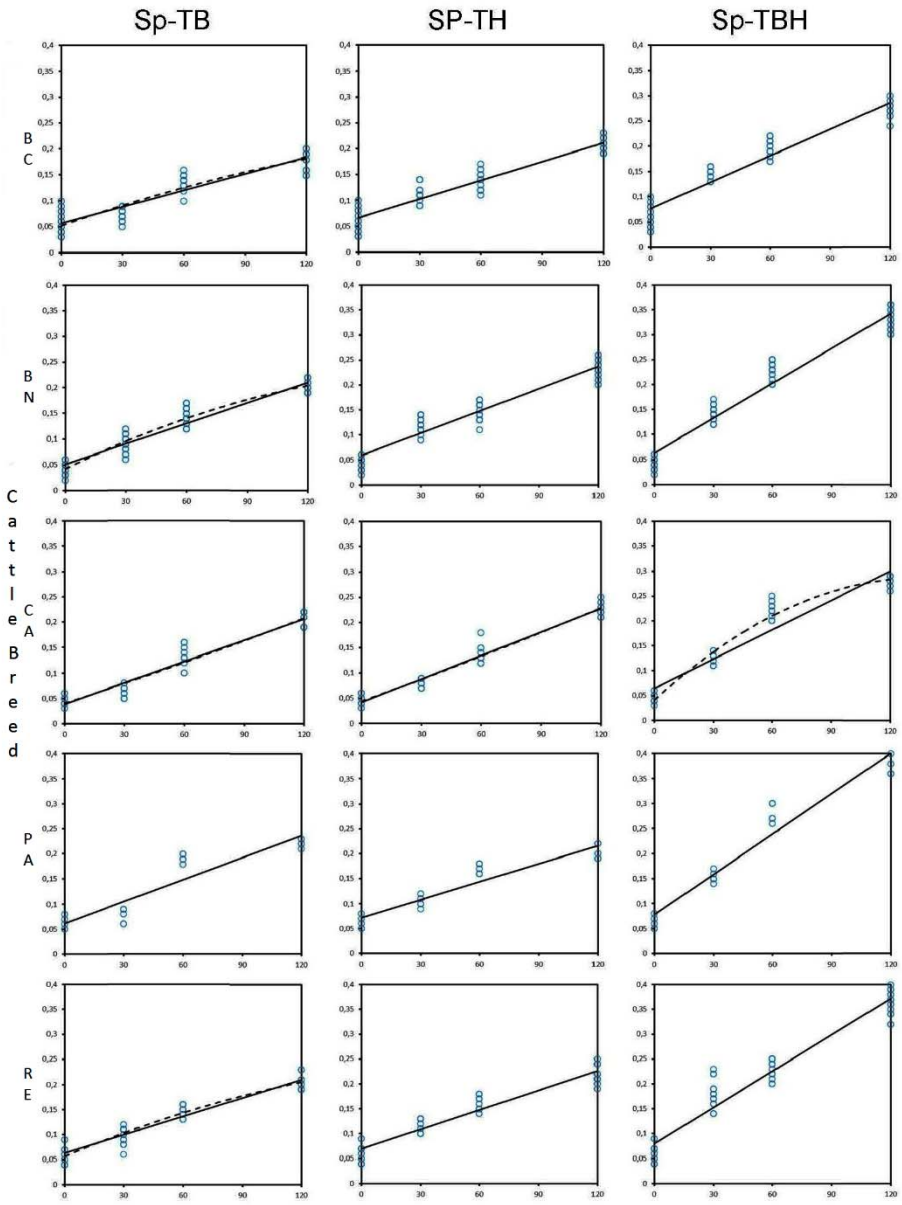
Culture Media (CM): Sp-T: Sp-TALP; Sp-TB: Sp-TALP + BSA; Sp-TH: Sp-TALP + Heparin and Sp-TBH: Sp-TALP + BSA + Heparin. Incubation

Time (IT): T30: 30m; T60: 60m and T120: 120m. Breed: BC: Berrendo en Colorado; BN: Berrendo en Negro; CA: Cardena Andaluza; PA: Pajuna and RE:Retinta. Values indicate the percentage of live acrosome-reacted spermatozoa expressed as means± S.E.

On each block, values with different letters (A,B,C and D) are significantly different (P<0.05, Duncan's test).

Figure 1: Two-way (breed versus culture media) orthogonal contrast analysis of results obtained in the acrosome reaction test of frozen-thawed bovine semen samples.

The percentage of acrosome reacted spermatozoa fits to a linear function over time in all analyzed breeds(full line, p<0.01). Some of these breed-media combinations (Sp-TB for BC, BN, CA and RE; Sp-TH for CA; and Sp-TBH for CA) were also fitted to a quadratic model (dotted line, p<0.05).



Culture Media: Sp-TB: Sp-TALP + BSA; Sp-TH: Sp-TALP + Heparin and Sp-TBH: Sp-TALP + BSA + Heparin.Breed: BC: Berrendo en Colorado; BN: Berrendo en Negro; CA: Cardena Andaluza; PA: Pajuna and RE:Retinta.

Discussion

Effective FIV still needs as a prerequisite a good functional status of spermatozoa which must retain their ability to complete the acrosome reaction in a timely manner. The inability of spermatozoa to undergo the AR affects the male fertilizing potential under both *in vivo* and *in vitro* conditions, and may play a decisive role in unexplained low fertility (Birck *et al.* 2010). It has been previously demonstrated in several species that the percentage of the *in vitro*-induced AR is significantly lower in subfertile animals compared with fertile animals (Whitfield and Parkinson 1992; Meyers *et al.* 1996; Herrera *et al.* 2002). For this reason, the study of this trait becomes important for high genetic value animals.

In general, the incidence of AR obtained *in vitro* in the current study is in consonance with those obtained by previous authors (Parrish *et al.* 1988a; Januskauskas *et al.* 2000; Somfai *et al.* 2002; Birck *et al.* 2010; Costa *et al.* 2010), indicating that doses of semen stored at Andalusian Genetic Bank responded satisfactorily to *in vitro* AR test. We also observed that the incidence of spontaneous AR at 0h was on average 6%. This result means that membrane damage due to freezing procedure was low in all studied samples, and thus they could be used in IVP programs (Kuroda *et al.* 2007). According with Coy *et al.* (2002), spontaneous acrosome-reacted spermatozoa are considered to be unable to fertilize oocytes. Moreover, the proportion of viable, uncapacitated spermatozoa present in semen has been previously shown to positively correlate with fertility in frozen-thawed semen samples (Thundathil *et al.* 1999) and may explain the appearance of clinical subfertility in cattle (Kuroda *et al.* 2007).

It is worth noting that the heparin added to culture medium increased the percentage of acrosome reacted spermatozoa in all studied breeds, as well as a synergistic effect was observed when heparin and BSA were added to culture medium (Sp-TBH). As documented by several authors (Parrish *et al.* 1988a; Dapino *et al.* 2006; Arangasamy and Singh 2007; Rodriguez *et al.* 2012), heparin has been shown to play a key role on *in vitro* sperm capacitation. Their initial contact with sperm induces the acrosome reaction by reorganizing membrane lipid domains, displacing spermatozoa surface proteins (Miller *et al.* 1990), stimulating the efflux of cholesterol and phospholipids, and increasing intracellular levels of calcium, pH and cAMP (Breininger *et al.* 2010). Moreover, cholesterol efflux has been identified as a fundamental proccess in the sperm acrosome reaction, resulting in an increase of the plasma membrane inestability (Cross 1998). On the other hand, BSA assists in that process accepting cholesterol released by spermatozoa when it undergoes into AR (Visconti and Kopf 1998; Kharche *et al.* 2009). In this sense, it has been previously demonstrated that the penetration rate of bovine oocytes processed in a protein free media was significantly lower than that obtained with BSA-supplemented media (Smetanina *et al.* 2006).

An unexpected result was that *in vitro* AR induced by the same incubation protocol was significantly different depending on the breed, suggesting differences between breeds in the response to AR test within cattle species. Although it has been previously demonstrated the influence of several paternal factors, such as male (Katska and Smorag 1996; Ward *et al.* 2001), age (Ward *et al.* 2002), breed (Söderquist *et al.* 1996), species (Pontes *et al.* 2010), and semen collection method (García-Álvarez *et al.* 2009) on FIV outcomes, this is the first attempt to

assess the effect of cattle breed on induced AR results. Early reports suggest that the ability of spermatozoa to undergo AR *in vitro* may be a useful method for predicting the result of FIV in cattle (Giritharan *et al.* 2005; Mukhopadhyay *et al.* 2008; Costa *et al.* 2010). In fact, AR status has been previously used as predictor of both freezability (Molnarova *et al.* 2006) and *in vivo* fertility (Collin *et al.* 2000; Giritharan *et al.* 2005; Birck *et al.* 2010) in cattle. However, it has been widely demonstrated that *in vitro* AR rates depend on both the incubation time (Fukui *et al.* 1990; Gliedt *et al.* 1996; Pavlok *et al.* 2001; Dode *et al.* 2002) and the culture medium (Sharma *et al.* 1999; Januskauskas *et al.* 2000; Vadnais *et al.* 2005). Our results are consistent with these previous observations, showing a clear interaction between culture medium and incubation time. However, these factors exerted differential effects on the AR status of bull spermatozoa for each of the studied breeds. This can partly be explained by breed differences in plasma membrane composition of bull spermatozoa (Casali *et al.* 1985). Due to this, the calculation of cattle fertility index from the outcome of AR test as suggested by previous authors (Molnarova *et al.* 2006; Mukhopadhyay *et al.* 2008; Costa *et al.* 2010) could not be recommended due to the effect exerted by breed, incubation protocol, and their interactions on the AR status of bull spermatozoa. Nevertheless, low AR rates could be a cause of unexplained infertility (Lessard *et al.* 2011).

Conclusions

Our results suggest that doses of stored semen of Andalusian cattle breeds respond satisfactorily to *in vitro* AR test, so that they could be used in future FIV programs. The response and pattern of the *in vitro*

AR-induced depend not only on culture medium and incubation time but also on the cattle breed. Therefore, all these factors must be taken in consideration to assess the rate of AR sperm in bulls. Further experiments are needed to validate AR test protocols in different cattle breeds for its use as an accurate predictor of *in vitro* fertility and as a valuable tool for calculating a fertility index (ranking) among bulls of different breeds.

Acknowledgements

Semen samples were gently donated by Berrendo (ANABE), Cardena Andaluza, Pajuna and Retinta breeders associations. Sebastian Demyda is supported by a grant from MAEC-AECID (Spain) and by University of Lomas de Zamora, Argentina (Project 13A178).

Capítulo III / Chapter III

"Effects of oocyte quality, maturation environment and incubation time on the number of chromosomal abnormalities in IVF-derived early bovine embryos"

Demyda-Peyrás, S., Dorado, J., Hidalgo, M., Anter, J., De Luca, L., Genero, E. & Moreno-Millán, M. 2012. **Effects of oocyte quality, incubation time and maturation environment on the number of chromosomal abnormalities in IVF-derived early bovine embryos.** Reproduction, Fertility and Development, *In Press - Published on-line-*.

Abstract

Chromosomal aberrations are one of the major causes of embryo failures in the mammals. It is also been demonstrated that the occurrence of this kind of abnormalities is higher on *in vitro* produced (IVP) embryos. This study was conducted to investigate the effect of oocyte morphology and maturation conditions on chromosomal abnormality rates in bovine preimplantational embryos. To this end, 790 early cattle embryos derived from oocytes with different morphology and maturated in different conditions (maturation period: 24 h *vs* 36 h; maturation media: five different serum supplements in TCM-199) were cytogenetically evaluated in three sequential experiments. Rates of normal diploidy and abnormal haploidy, polyploidy and aneuploidy were determined in each embryo. Throughout all experiments, the rate of chromosomal abnormalities were significantly ($p<0.05$) influenced by the oocyte morphology and maturation conditions (maturation time and culture media). We find that a lower morphological quality is connected with high rates of numerical chromosome abnormalities ($p<0.05$). Moreover, we also find that polyploidy is associated with increased maturation time ($p<0.01$), whereas maturation media significantly ($p<0.05$) affected the rates of haploidy and polyploidy. In general, the use of ECS or FCS as serum supplements produced higher rates of chromosomal aberrations ($p<0.05$) compared to the other serum supplements. According to our results, we can conclude that morphological quality of oocytes and maturation conditions affect the chromosomal abnormality rates of the subsequent IVP cattle embryos.

Keywords: Chromosomal abnormalities, *in vitro*, bovine embryo, oocyte maturation

Introduction

The bovine *in vitro* fertilization (FIV) industry has been constantly improving during the last decade. At present time the number of *in vivo* and *in vitro* produced (IVP) embryos is similar in some countries (Pontes *et al.* 2010). Despite the vast researches performed during the last years, the overall efficiency of IVP cattle embryos still remains low. Only 30% of cultured oocytes reach the blastocyst stage (Lonergan 2007). It is well known that *in vivo* derived embryos have higher quality and viability to term than those produced by *in vitro* maturation, fertilization and culture (Ulloa Ulloa *et al.* 2008b), even when the same donor cow was used (Pontes *et al.* 2009). While the causes of this inefficiency are likely numerous and complex, the oocyte morphological quality at the beginning of the IVP process and the oocyte maturation protocols have been described as major contributing factors on this issue. Several authors demonstrated that oocytes with higher morphological quality (class "A" and "B") have shown improved developmental rates to term in the subsequently formed embryos (Hawk and Wall 1994). In this sense, maturation media (Bilodeau-Goeseels 2006; Räty *et al.* 2011), maturation protocols (Yang *et al.* 1993) and serum supplementation (Fukui and Ono 1989; Pereira *et al.* 2010) are also described as potential causes of these differences. For this reason different *in vitro* oocyte maturation media, maturation protocols and media supplementation patterns are being developed and tested constantly in order to meet the metabolic requirements just as *in vivo* conditions (Rizos *et al.* 2002; Purpera *et al.* 2009).

Multiple causes have been described as being responsible for increased developmental failure on IVP embryos (Van Soom *et al.* 2007;

Vandaele and Van Soom 2011). In this sense, chromosomal abnormalities are thought to be a major contributor and are associated with reduced developmental potential (King *et al.* 2006), impaired embryo viability, (Kawarsky *et al.* 1996) and embryonic death and abortion (King 1990). In cattle, it has been reported that IVP embryos have a higher rate of chromosome abnormalities than their *in vivo* counterparts (Viuff *et al.* 1999). Similar results have been found in pig (Ulloa Ulloa *et al.* 2008a), horse (Rambags *et al.* 2005), goat (Villamediana *et al.* 2001) and sheep (Alexander *et al.* 2005). It has been demonstrated that the percentage of chromosomal abnormalities appears to be significantly influenced by maturation environment in oocytes (A'Arabi *et al.* 1997) and by post-fertilization culture conditions on IVP embryos (Lonergan *et al.* 2004).

Currently, it is not possible to evaluate the chromosomal complements of matured oocytes previous to fertilization in the FIV laboratory by simple and non-invasive techniques (Coticchio *et al.* 2004). Therefore, oocytes with normal maturation parameters can have an abnormal chromosomal composition, producing higher rates of fertilization failures and development impairment. Despite of their potential economic impact on livestock production, only a few cytogenetic studies of IVP embryos have been reported (Garcia-Herreros *et al.* 2010). Moreover, moreover they did not focus on the influence of oocyte quality and maturation environment as potential causes of these alterations.

The present study was therefore designed to examine the effect produced by: 1) the oocyte morphological quality, 2) oocyte maturation time, and 3) oocyte maturation media (serum supplementation), on the

incidence of numerical chromosomal abnormalities in early IVP bovine embryos.

Materials and methods

Media and reagents

Chemicals were purchased from Sigma-Aldrich, Spain, unless otherwise is indicated. Percoll were purchased from GE Healthcare, Spain. Nunclon™ Plastic dishes were purchased from Termofischer Scientific, Germany. Sterilization filters used in culture media were purchased from Millipore Corporation, Spain. Oocyte and embryo were manipulated with 20 µl micropipette tips purchased from Eppendorf Iberica, Spain. Estrus cow serum (ECS) were obtained according to our previous protocols (Ocana-Quero *et al.* 1999b). Briefly, sterile complete blood samples were obtained by jugular venopunction of six estrous cows. Pooled serum was obtained by centrifugation (200 x G, 15 min), inactivated by heat (56ºC, 30 min) and sterilized with a 0.22 µm membrane filter. Aliquots were frozen and stored at -20ºC until its use. Anestrus cow serum (ACS) was obtained following the same protocol by collecting blood samples from 6 different anestrous cows.

In vitro embryo production

Bovine embryos were produced according to previous studies performed in our laboratory (Ocana-Quero *et al.* 1999a), with the following modifications:

Collection of ovaries and oocytes and in vitro maturation

Ovaries were collected from slaughtered cows in the local abattoir and transported to the laboratory within the next 2 h after collection. The ovaries were washed thrice in warm physiological saline solution supplemented with kanamycin (25 mg/ml). Cumulus oophorus complexes (COCs) were obtained from follicles between 4 and 8 mm through aspiration with 18 G needle and placed into a 15 ml conical tube with 2 ml of warm H-TALP media. After 1 h sedimentation at 38.5ºC, oocytes were poured on Petri dishes and examined under a stereomicroscope with a warm plate. In Experiment 1, oocytes were classified according to their morphology in four groups (A, B, C and D) (Hawk and Wall 1994; Cetica *et al.* 1999). Based on the outcome of Experiment 1, and to eliminate the possible detrimental effect of morphological quality, only class "A" oocytes (compact-intact cumulus cell layers and good morphology) were used for Experiments 2 and 3 of this study. Selected COCs were washed twice in H-TALP and cultured in groups of 100 in 500 µl modified bicarbonate-buffered TCM199 media, supplemented with 10% of fetal calf serum (FCS); 0.4 mmol/l glutamine; 0.2 mmol/l sodium pyruvate; 0.5 mg/ml FSH, 5 mg/ml LH; 1 mg/ml estradiol and gentamicin (50 mg/ml) for 24 h, at 38.5ºC in 5% CO_2.

Sperm capacitation and in vitro fertilization

Frozen semen straws from different bulls of the Retinta breed with a history of proven fertility were thawed in a water bath at 38ºC during 1

min. Viable spermatozoa were selected by centrifugation through a discontinuous Percoll gradient (45 and 90% (v/v) in Sp-TALP) and washed twice in warm equilibrated Sp-TALP media. The final sperm concentration of 1 x 10^6 sperm cells/ml was adjusted in IVF-TALP, supplemented with bovine serum albumin (BSA; 6 mg/ml) and heparin (100 mg/ml). Groups of 60 oocytes were washed in 500 µl of equilibrated IVF-TALP and incubated with sperm at 38.5ºC in 5% CO_2.

Culture of zygotes

After 20–24 h of incubation, groups of 100 presumptive zygotes were denuded by vortex in 15 ml tubes during 3 min. Thereafter, it were transferred to synthetic oviduct fluid (SOF), supplemented with BME amino acid solution and MEM non-essential amino acid solution and 5% (v/v) FCS and were cultured in 50 µl droplets under mineral oil in groups of 25 for 72 h at 38.5ºC in 5% CO_2.

Chromosome preparation

Chromosome preparation was performed according to our standard method (Ocana-Quero *et al.* 1999a). At the end of the culture period, colchicine (0.1 µg/ml) was added to the culture media and embryos were cultured for up to 6 h. After that, embryos were placed in a 500 µl hypotonic solution of 0.88% (w/v) tri-sodium citrate for 45–60 min. Thereafter, embryos were fixed mildly by pouring about 500 µl of Carnoy´s 1:1 fixative (one part acetic acid: one part methanol) for 10 min and placed in a 3:1 Carnoy´s fixative overnight. Each embryo was then fixed onto a clean glass slide and blastomeres were separated using acetic

acid (100%, v:v). The nucleus from each blastomere was spread with several drops of Carnoy´s 1:1 fixative. Samples were air-dried and stained with 5% Giemsa solution for 10 min. Only those blastomeres containing an analyzable metaphase (intact and non-overlapping chromosomes) were assessed under X 1250 magnification in an optical microscope (Reichert-Jung Polyvar, Austria).

Cytogenetic criteria

Only the embryos showing more than one analyzable metaphases were included in this study. Embryos with 60 chromosomes in all countable metaphases were defined as normal diploids (2n = 60). Those in which all analyzable metaphases contained 30 chromosomes were defined as haploids. If the embryo have shown more than two sets of chromosomes (3n = 90 or 4n=120) in all blastomeres were judged as polyploids. Finally, those that showed an abnormal chromosomal number were defined as aneuploids.

Experiment 1

The aim of this experiment was to examine the incidence of morphological oocyte quality on the appearance of chromosomal abnormalities in early IVP bovine embryos. A total of 249 oocytes were classified in four different groups (A, B, C and D) according to their subjective quality as described previously. Oocytes were fertilized, cultured and cytogenetically analyzed as described above.

Experiment 2

The aim of this experiment was to evaluate the incidence of oocyte maturation time on the appearance of chromosomal abnormalities in early IVP bovine embryos. In this study, a total of 157 embryos derived from class "A" morphological quality oocytes were classified into two groups according to their maturation period: 24 h and 36 h. Thereafter, oocytes were fertilized, cultured and cytogenetically analyzed as described above.

Experiment 3

This experiment was performed to investigate the effect of different serum supplementation strategies during *in vitro* oocyte maturation on the percentage of chromosomal abnormalities observed in early IVP bovine embryos. A total of 384 embryos derived from class "A" morphological quality oocytes were produced as described previously from oocytes matured in 6 groups of TCM-199 medium supplemented with: 10% ECS (n = 62; prepared as described previously); 10% FCS (n = 59; F2442, Sigma Aldrich, Spain); 10% ACS (n = 59; prepared as described previously); 10% of bovine amniotic fluid (BAF; n = 60; A5130, Sigma Aldrich, Spain); 0,6% (w/v) BSA (n = 74) and 10% of bovine steer serum (BSS; n = 70; A9433, Sigma Aldrich, Spain) used as control. After maturation, oocytes were fertilized, cultured during 72 h and cytogenetically analyzed as described previously.

Statistical analysis

Data were analyzed using the Chi-Square procedure of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). Differences observed among treatments were determined using a two factors expected proportion test. Data were significant at $p<0.05$.

Results

Significant ($p<0.05$) effects of oocyte morphological quality, oocyte maturation time and media serum supplementation were found on the percentage of abnormal chromosomal complements observed across all the analyzed embryos.

Experiment 1

A significant ($p<0.05$) relationship between the oocyte quality and the ploidy of the resulting embryos was found (Table 1). The number of normal diploid embryos significantly ($p<0.05$) increased with increasing oocyte morphological quality of the oocyte. Therefore, the lowest percentage of total chromosomal abnormalities ($p<0.05$) were observed only in embryos derived from superior quality oocytes (class "A"). When analyzing separately each kind of chromosomal abnormalities, we found that the highest haploidy percentage ($p<0.05$) was observed in embryos derived from oocytes class "C" and "D". Similarly, the percentages of polyploidy and aneuploidy were statistically ($p<0.05$) lower in embryos

derived from class “A” oocytes compared to those classified as class “B”, “C” and “D”.

Table 1: Analysis of chromosomal complements of day 3 IVP bovine embryos derived from oocytes classified according their morphological quality

Oocyte Quality	Total number of analyzed embryos	Chromosomal complements											
		Normal Embryos		Abnormal embryos									
		Diploid		Total		Haploid		Polyploid		Aneuploid			
A	60	56	93,33% [a]	4	6,67% [c]	1	1,67% [bc]	2	3,33% [b]	1	1,67% [b]		
B	65	53	81,54% [ab]	12	18,46% [b]	2	3,08% [b]	6	9,23% [ab]	4	6,15% [ab]		
C	61	45	73,77% [bc]	16	26,23% [ab]	6	9,84% [ab]	6	9,84% [ab]	4	6,56% [ab]		
D	63	39	61,90% [c]	24	38,10% [a]	10	15,87% [a]	8	12,70% [a]	6	9,52% [a]		

On each row, values followed by different letters (a, b and c) show statistical significant differences (P<0.05, Two tailed Z-score test).

Experiment 2

Chromosomal abnormality rates were significantly ($p<0.01$) higher in oocytes matured for 36 h than those matured for 24 h (Table 2). Interestingly, only the polyploidy rates were significantly ($p<0.01$) different between treatments. However, no significant ($p>0.05$) differences were found between the other type of chromosomal abnormalities (haploid and aneuploid) and maturation time (24 h and 36 h).

Table 2: Analysis of chromosomal complements of day 3 IVP bovine embryos from oocytes maturated by two different periods

		Chromosomal complements									
		Normal Embryos	Abnormal embryos								
Oocyte maturation time (hs)	Number of embryos analyzed	Diploid	Total		Haploid		Polyploid		Aneuploid		
24	77	71	92,21%	A	6	7,79%	B	2	2,60	a	3	3,90%	A	1	1,30%	a
36	80	60	75,00%	B	20	25,00%	A	6	7,50	a	12	15,00%	B	2	2,50%	a

On each row, values followed by different capital letters (A and B) show highly significant differences (P<0.01, Two tailed Z test for proportions).

Experiment 3

Supplementation strategies of maturation media with different serum significantly ($p<0.05$) influenced the incidence of chromosomal abnormalities of IVP bovine embryos (Table 3). In general, the use of ECS or FCS as serum supplements produced higher rates of chromosomal abnormalities ($p<0.05$) compared to the other serum supplements. Moreover, the rates of haploidy and polyploidy were significantly ($p<0.05$) higher in those embryos maturated in the presence

of ECS. However, there was no statistical ($p>0.05$) differences between supplements for the rate of aneuploidy.

Discussion

Chromosomal abnormalities have been suggested as a major cause of embryonic failure on IVP embryos (King *et al.* 2006). Previous reports demonstrated that the incidence of these abnormalities appears to be influenced by methods of embryo handling used during these procedures as well as suboptimal culture environments in which embryos must be developed (King *et al.* 2006). The present study evaluated the impact of the quality of the oocytes and their *in vitro* maturation environment on the chromosome make-up of the subsequently produced embryos, which is one of those potential causes described above. In this way, our results showed that initial poor quality of oocytes as well as suboptimal maturation conditions produced a higher rate of chromosome abnormalities at earliest stages of embryonic development.

Table 3: Analysis of chromosomal complements of day 3 IVP bovine embryos from oocytes maturated in different culture media

Culture media Suplementation	Number of embryos analyzed	Chromosomal complements											
		Normal Embryos		Abnormal embryos									
		Diploid		Total		Haploid		Polyploid		Aneuploid			
BSS	70	66	94,29% [a]	4	5,71% [c]	2	2,86% [b]	1	1,43% [b]	1	1,43% [a]		
ACS	59	54	91,53% [a]	5	8,47% [c]	1	1,69% [b]	3	5,08% [b]	1	1,69% [a]		
BAF	60	54	90,00% [abc]	6	10,00% [bc]	2	3,33% [b]	2	3,33% [b]	2	3,33% [a]		
BSA	74	66	89,19% [ab]	8	10,81% [bc]	5	6,76% [ab]	3	4,05% [b]	0	0,00% [a]		
FCS	59	46	77,97% [bcd]	13	22,03% [b]	5	8,47% [ab]	6	10,17% [ab]	2	3,39% [a]		
ECS	62	37	59,68% [d]	25	40,32% [a]	11	17,74% [a]	12	19,35% [a]	2	3,23% [a]		

On each row, values followed by different letters (a, b and c) show statistical significant differences (P<0.05, Two tailed Z-score test).

BSS: bovine steer serum; ACS: anestrous cow serum; BAF: bovine amniotic fluid; BSA: bovine serum albumin; FCS: fetal calf serum and ECS: estrous cow serum

The first experiment was conducted to evaluate the importance of oocyte morphology in the incidence of chromosomal abnormalities in subsequent produced embryos. Previous studies demonstrated that the initial quality of the oocytes had greater impact on early embryonic survival, establishment and maintenance of pregnancy (Krisher 2004). In our study, the rate of chromosome abnormalities in embryos was significantly affected by the morphological quality of the oocytes. In this sense, it has been also demonstrated that the maturation rates and embryo development of the subsequent produced embryos are strongly related with the oocyte original morphology (Long *et al.* 1994; Blondin and Sirard 1995). However, to our knowledge, there are no previous studies that evaluate the chromosomal composition of the derived embryos of morphological classified oocytes as possible cause of these losses in IVP cattle embryos. In our study, we observed in matured oocytes that begin to multiply after fertilization higher rates of chromosomal abnormalities associated with low quality oocytes (pale and heterogeneous cytoplasm, class "D"). As a possible explanation, this type of oocytes has a low density of organelles to support the meiotic process appropriately that leads to higher rates of meiotic failures (Nagano *et al.* 2006). On the other hand, the lower incidence of morphologically normal spindles in the low quality oocytes leads to higher rates of chromosomal gains or losses during replication (Long *et al.* 1994). In this way, embryos derived from lower-grade oocytes, with poor cytoplasmic quality (granularity or discoloration of the cytoplasm, vacuolization, and incorporations such as refractile bodies), are related with pregnancy rates which are five times lower than those of embryos derived from normal quality oocytes(Ebner *et al.* 2003). We would like to highlight the fact that only high quality oocytes (class "A") showed a lower polyploidy rate. Recent studies have

shown that the incidence of polyspermic fertilization, major cause of embryo polyploidy, is higher in low quality oocytes compared to those with optimal morphology (Wortzman and Evans 2005). This fact can be attributed to a delayed and incomplete exocytosis of the cortical granules (Wang *et al.* 1997) or to a premature cortical granules release in low quality oocytes (Suzuki *et al.* 2003), causing a weaker block to polyspermy.

In Experiment 2, embryos derived from high quality oocytes (class "A") were differently matured and cultured *in vitro*. A higher rate of chromosomal alterations was observed when the oocyte maturation time was increased from 24 to 36 h. It has been widely demonstrated that longer maturation times decreases the outcome of IVP embryos (Gliedt *et al.* 1996) and stimulate higher nuclear maturation rates by a greater positive feedback from cumulus cells (Barrett and Albertini 2010). However, over-maturation for more than 24 h promotes an "aging" effect, showing signs of degeneration probably due to the excessive time in sub-optimal maturation environment (Máximo *et al.* 2012). An interesting observation was that only the rates of polyploidy were significant higher when the time of maturation was increased. This abnormality was noted as a major cause of developmental failure on IVP embryos in mammals. It has been previously suggested that maturation prepares the oocyte to interact with the fertilizing spermatozoa at a particular moment in time (Dale and Defelice 2011). If the oocyte is not fertilized, the maturation processes continue and the cell ages, leading to higher polyspermy rates (Tarín 1996). In a later study, Tarín *et al.* (1999) also suggest that oocyte post-ovulation aging may leads to a series of ultrastructural changes related to the meiotic spindle integrity. Moreover, same authors suggest that these changes may produce higher rates of

failure in the extrusion of the second polar body by the oocyte after sperm penetration, leading to higher polyploidy rates. Polyspermy has been widely described as a major concern particularly in pig embryos (Niwa 1993). Rather, partial induction of the acrosome reaction of boar spermatozoa in FIV media and sperm fertilizing doses have been indicated as the primary causes in this species (Funahashi 2003).

Previous authors have demonstrated the influence of the culture system upon the ploidy of *in vitro* matured oocytes in human (Christopikou *et al.* 2010) and domestic animals (Ocana-Quero *et al.* 1999b; Lechniak *et al.* 2005; Ueno *et al.* 2005). Furthermore, the same effect was observed in oocyte nuclear maturation rates and blastocyst yield of IVF-derived embryos in goat (Tajik and Esfandabadi 2003), porcine (Ott *et al.* 2002) and cattle (Ocana-Quero *et al.* 1999b; Russell *et al.* 2006a). As a possible explanation, age related changes in oocytes due to oxidative damage associated with high cellular metabolism and genomic instability, (e.g., mitochondrial mutations and telomere shortening) can be triggered by the environmental culture conditions (Pan *et al.* 2008). Mainly, this can be due to a lack of energy supply by oxidative phosphorylation in the mitochondria within the oocyte (Eichenlaub-Ritter *et al.* 2011). Our study is in agreement with theses hypothesis, since we found a clear influence of the culture media in the ploidy of the derived embryos. However, it has been described that matured oocytes appear fairly normal until resumption of maturation when they form aberrant spindles, without being able to distinguish them from the normal (Ebner *et al.* 2003). Since we cannot evaluate the chromosomal composition and meiotic spindle previous to fertilization, it can be assumed that oocyte with higher level of chromosomal abnormalities, produced by the suboptimal environmental conditions

(A'Arabi *et al.* 1997), will produce embryos with an higher rate of abnormalities that can have a normal development at very early stages (King *et al.* 2006).

In Experiment 3, the higher rates of chromosomal abnormalities were observed in the culture medium supplemented with ECS. It has been demonstrated that this serum supplementation produces higher levels of FSH, LH and estrogens in the maturation media (Ott *et al.* 2002). These hormones have been associated with oocyte aging (Liu *et al.* 2011), marked ultrastructural changes of embryonic cells and with higher rate of blastomeric apoptosis (Ott *et al.* 2002) and chromosomal abnormalities (Vialard *et al.* 2011). Indeed, embryos derived from these oocytes can develop aberrant mitotic spindles, leading to a failure of chromatid migration to the cell poles during cell division, DNA replication without associated cytokinesis and consequently to an altered ploidy (Liu and Keefe 2002).

The lack of a significant difference in aneuploidy rates in the results of Experiment 2 and 3 suggests that maturation environment (oocyte maturation time and culture media supplementation) is less involved in the appearance of this kind of chromosomal abnormalities. In fact, aneuploidy have mainly been related to intrinsic factors of the humans (Frumkin *et al.* 2008) and bovine oocytes (Lechniak *et al.* 1996; Nicodemo *et al.* 2010).

Previous authors suggest that post-fertilization culture environment of the developing embryo can affect the incidence and severity of chromosomal abnormality rates in the resulting blastocyst (Lonergan *et al.* 2004; Ulloa Ulloa *et al.* 2008b; Xu *et al.* 2008). However, all of these studies evaluated the chromosomal complements of derived embryos at blastocyst stage. Dieleman *et al.* (2002) suggest that the ploidy of the

resulted embryos is less affected at early stages of development by the culture environment. It has previously been described (Tajik *et al.* 2008; Mermillod *et al.* 2010) that long-term incubation induce developmental failures in embryos due to their exposure to higher levels of toxic metabolites and oxidative stress in late culture developmental stages.

Conclusions

Morphological quality of oocytes and maturation protocols affect the chromosomal abnormality rates of the subsequent IVP cattle embryos. Further studies are necessary to improve the oocyte maturation protocols currently used in the *in vitro* cattle embryo production industry to minimize the appearance of chromosomal abnormalities on the subsequent produced embryos.

Acknowledgements

We thank to the COVAP slaughterhouse (Pozoblanco, Spain) by provides the ovaries for this study. We also thank to Dr. Ann Van Soom, Ms. Petra Van Damme and Ms. Isabel Lemahieu for their excellent technical assistance. Sebastian Demyda is supported by a grant from MAEC-AECID (Spain). This study was supported by the University of Cordoba, Spain.

Discusión general / General discussion

Discusión general

Debido al formato utilizado en la redacción de la presente tesis doctoral, los resultados de los distintos experimentos han sido discutidos ampliamente a lo largo de los respectivos capítulos de esta tesis. En ellos se han tratado de explicar las posibles causas y efectos que hemos observado en los resultados obtenidos durante el desarrollo experimental. El objetivo de esta discusión general es analizar todas las discusiones previas de una manera global.

Durante nuestra experimentación hemos podido observar que existen numerosos pequeños factores que pueden influir en el éxito o en el fracaso de un programa de FIV. Esto ya había sido descrito en numerosas revisiones realizadas (Hansen 2006; Lonergan 2007). Similares conclusiones se ha obtenido durante el análisis de los resultados observados en la producción comercial de embriones bovinos *in vitro* Brasilera, en donde se producen más del 80% de los embriones FIV a nivel mundial (Pontes *et al.* 2010).

Entre estos pequeños factores nuestro trabajo ha logrado reafirmar la importancia que los efectos genéticos tienen sobre los porcentajes de eficiencia obtenidos. Curiosamente, este aspecto no ha sido estudiado en profundidad hasta la fecha en el ganado bovino mediante un enfoque sistemático. Si bien existen datos al respecto, como fue mencionado en la introducción, la mayoría de ellos han sido obtenidos analizando los datos resultantes de los programas comerciales que se llevan a cabo en algunas razas en particular. (Camargo *et al.* 1997a; Hernández-Cerón *et al.* 2004; Gandini *et al.* 2007; Getz *et al.* 2008). Una posible explicación sería que estas empresas privadas cuenten con protocolos de adaptación de sus

técnicas al tipo de animal utilizado, pero que se mantengan en reserva por una cuestión de política empresaria.

Las diferencias observadas en las tasas de maduración ovocitaria *in vitro* han sido atribuidas a diversos factores. Entre ellos podemos citar a la morfología original del ovocito y al folículo en el cual los ovocitos se han desarrollado (Blondin and Sirard 1995). También se ha demostrado que el número de ondas foliculares existentes durante el ciclo sexual del animal donante (Domínguez 1995), la raza del animal empleado (Santos *et al.* 2008; Abraham *et al.* 2012) y el número de ovocitos obtenidos en cada animal (Ferreira *et al.* 2009) pueden influir en la eficiencia general del proceso madurativo. Todos estos factores, que tienen una clara base genética, son producto de un proceso de selección que no ha tenido en cuenta este tipo de caracteres. Esto puede deberse bien a un esquema antrópico como el utilizado en las razas comerciales o bien a la selección natural que se da normalmente en las poblaciones silvestres. Como consecuencia de ello se ha observado en uno u otro caso una falta de homogeneidad en los resultados obtenidos en las distintas razas. Estas diferencias, observadas principalmente en razas de uso intensivo, podrían deberse la alta presión de selección focalizada en los caracteres productivos en menoscabo de los caracteres generales, como pueden ser la rusticidad o la fertilidad de los rebaños (Guyader-Joly *et al.* 2009; Panetto *et al.* 2010). Nuestros resultados se hallan en consonancia con investigaciones previas, demostrando que el comportamiento de los ovocitos y de las células espermáticas de las distintas razas españolas analizadas no es homogéneo. Por otra parte, en el caso de los gametos femeninos, la baja variabilidad genética que se observa en el Toro de Lidia después de 400 años de selección únicamente en virtud a su bravura (Cortés *et al.* 2011), podría tener su reflejo en una baja tasa de

recuperación de ovocitos de cada ovario procesado, como hemos observado en nuestros estudios, pero no así en su maduración y fertilización *in vitro*.

Similares resultados se han obtenido cuando analizamos a las razas bovinas andaluzas en peligro de extinción, en las cuales existen diferencias importantes en la variabilidad genética observada en las diferentes ganaderías debido a la escasa cantidad de animales existentes en cada una de ellas (Rodero *et al.* 2011). Hemos podido determinar que el comportamiento de las células espermáticas capacitadas *in vitro* demuestra un patrón diferente en cada raza evaluada, máxime cuando son utilizados diferentes tiempos y medios de cultivo. El análisis de los datos obtenidos nos ha demostrado que los resultados no son comparables entre las distintas razas, aunque todas ellas hayan respondido de forma satisfactoria al test de inducción acrosómica. Este experimento sugiere que, ante la ausencia de otros factores discriminantes, estas diferencias observadas se deban probablemente a la variabilidad genética interracial. Existen estudios previos que sugieren que los resultados obtenidos con el uso del test de capacitación *in vitro* (test de reacción acrosómica) pueden ser correlacionados de manera directa con la fertilidad potencial de los animales para su uso en la inseminación artificial (Giritharan *et al.* 2005; Birck *et al.* 2010) o en la FIV (Molnarova *et al.* 2006). Sin embargo, y según nuestros resultados, este test no debería utilizarse para clasificar animales pertenecientes a distintas razas.

Finalmente, nuestro estudio se ha centrado en la evaluación de las posibles causas de las anomalías cromosómicas de los embriones producidos mediante FIV. Los datos obtenidos en los seres humanos, en donde se realizan la mayor cantidad de investigaciones en este campo, suelen relacionar este tipo de anomalías principalmente a los ovocitos

(Munné *et al.* 1998; Munné *et al.* 2007). Esta influencia ovocitaria ha sido atribuida a diversos factores como la edad materna, la morfología embrionaria (Munné *et al.* 2007) y el medio de maduración utilizado (A'Arabi *et al.* 1997). Nuestros resultados han confirmado que el efecto que estos factores producen en la bajada de la eficiencia y el aumento de la tasa de perdidas embrionarias en la FIV bovina (Knijn *et al.* 2002; Park *et al.* 2005; Russell *et al.* 2006a) se debe, al menos parcialmente, a la influencia de los mismos en el aumento de la cantidad de embriones cromosómicamente aberrantes. Nuestro trabajo experimental ha demostrado también que las incidencias de los diversos tipos de anomalías cromosómicas se ven afectadas dependiendo de los factores arriba mencionados. Así hemos observado que las haploidías y las hiperploidías son profundamente dependientes de la morfología celular, del tiempo de maduración y del medio de cultivo empleado. Sin embargo estos mismos factores no tienen la misma influencia sobre las de aneuploidías embrionarias. Esto podría deberse a la inducción de un "envejecimiento ovocitario" temprano, el cual produce fallos en la estructura micro-tubular del ovocito en desarrollo, favoreciendo la aparición de este tipo de anomalías debido a errores en la disyunción cromosómica. Por lo tanto será necesario tener en cuenta estos factores a la hora de ajustar los protocolos de fertilización *in vitro* en el bovino, limitando así la aparición de estas anomalías.

General discussion

Due to the arrangement used in this thesis, based on article´s compendium, each experiment performed has been widely discussed the respective chapters of this thesis. In these individual discussion´s we have tried to explain the possible causes and effects of the results obtained in each one of the experiments performed. Accordingly, the objective of this general discussion is to analyze all previous discussions on a global basis.

During experimental development we have seen that there are many "small factors" influencing the success or failure of an IVF program. This has been mentioned in numerous reviews previously written in this topic (Hansen 2006; Lonergan 2007). However, the same conclusion can be drawn from the analysis of the results obtained in the Brazilian commercial production of *in vitro* bovine embryos, where more than 80% of the IVF embryos worldwide are produced (Pontes et al. 2010).

Among these factors, our work has been able to reaffirm the importance of the genetic effects on the efficiency rates achieved during each particular IVF program. Interestingly, this aspect was not widely studied in cattle using a systematic approach. Although there are some reports in this regard, as mentioned in the introduction, most of them are made by analyzing data from commercial programs that are conducted in certain breeds. (Camargo et al. 1997; Hernandez-Ceron et al. 2004; Gandini et al. 2007; Getz et al. 2008). However, we suggest that it is possible that companies conducting these programs have more information regarding the adaptation of their protocols and techniques to the type of animal used but it are kept in reserve as a matter of corporate policy.

The differences observed among *in vitro* oocyte maturation rates have been attributed to several factors. Among them we can mention the morphology of the oocyte and the follicle in which the oocyte has been developed (Blondin and Sirard 1995). It has also been shown that the number of follicular waves produced during the sexual cycle of the donor animal (Dominguez 1995), the breed of animal used (Santos et al. 2008; Abraham et al. 2012) and the number of oocytes obtained in each animal (Ferreira et al. 2009) can influence the overall efficiency of the *in vitro* maturing process. All these factors, which have a clear genetic basis, are the result of a selection process that did not considered this type of characters. This may well be due to anthropic selection scheme, as used in commercial breeds, or due to natural selection that occurs normally in wild populations, producing, in both cases, non homogeneous results among breeds. These differences, observed principally in animals with an intensive production system could be mainly due to the high selection pressure focused on production traits at the expense of the general characteristics, such as rusticity or fertility (Guyader-Joly et al. 2009; Panetto et al. 2010). Our results are consistent with previous researches, showing that the *in vitro* behavior of oocytes and sperm cells of different breeds studied differs among them. In the case of the female gametes, the low genetic variability observed in the Lidia breed after 400 years of selection only under his bravery (Cortés et al. 2011) could be reflected in a low number of oocytes obtained from each processed ovary, but not in *in vitro* oocyte development.

Similar results were obtained when analyzing the Andalusian endangered cattle breeds, in which there are important differences in their genetic variability in among herds due to their small number of animals (Rodero et al. 2011). We observed that the behavior of *in vitro*

capacitated sperm cells has a different pattern on each breed evaluated when used different culture times and culture media. The analysis of the data has shown that, although all of them have responded satisfactorily to acrosome induction test, the results are not comparable between the different breeds. This experiment suggests that, in absence of other discriminating factors, the observed differences may be due to interbreed genetic variability. Previous studies suggest that the use of *in vitro* capacitation (acrosome reaction test) allows classification of the animals upon fertility for its use in artificial insemination (Giritharan et al. 2005; Birck et al. 2010) and IVF (Molnarova *et al.* 2006). However, according to our results, it has should not be used to classify animals belonging to different breeds.

Finally, our study has also focused on the evaluation of the possible causes of chromosome abnormalities in embryos produced by IVF. Previous research, conducted mostly in humans, have primarily related such anomalies to the oocytes (Munné *et al.* 1998; Munné *et al.* 2007). This influence oocyte has been attributed to various factors such as maternal age, embryo morphology and culture environment (A'Arabi *et al.* 1997). Our results confirmed that part of the influence of these factors on the low efficiency and increased embryonic loss rates observed in bovine IVF (Knijn et al. 2002; Park et al. 2005, Russell et al. 2006) can be explained by an increase in the number of embryos with aberrant chromosome complements. The experimental work performed has also shown that each kind of chromosomal abnormalities are differentially affected by the factors studied. We have shown that hiperploidies and haploidies are dependent on cell morphology, culture time and the culture medium used. Oppositely, this was not observed in the case of the aneuploidies. This could be due to the induction of an "oocyte aging"

effect which produces early failures in the micro tubular structure of the oocyte, promoting the appearance of such anomalies mostly due to errors in the chromosome disjunction. It will therefore be necessary to consider the factors that could affect results adversely when the protocols for *in vitro* fertilization in cattle are designed to limit the occurrence of these anomalies.

Conclusiones / Conclusions

Conclusión general

Los resultados obtenidos en la producción de embriones bovinos mediante la fertilización *in vitro* están influidos por la raza de los animales utilizados y por los porcentajes de anomalías cromosómicas derivadas de los protocolos utilizados.

Conclusiones particulares:

1. Los ovocitos pertenecientes a vacas de la raza de Lidia pueden ser obtenidos, madurados y fertilizados utilizando protocolos estandarizados de MIV y FIV.
2. El número total de COCs y ovocitos viables recuperados de ovarios pertenecientes a vacas de Lidia es menor al obtenido en otras razas bovinas estudiadas previamente.
3. La suplementación hormonal del medio de maduración ovocitaria mejora los porcentajes de ovocitos maduros sin afectar el proceso de fertilización *in vitro.*
4. Las dosis existentes de semen crio-conservado pertenecientes a las cuatro principales razas bovinas Andaluzas en peligro de extinción han respondido satisfactoriamente al test de inducción acrosómica *in vitro*, posibilitando su uso en futuros programas de FIV.
5. El patrón de respuesta del test de inducción acrosómica *in vitro* depende no sólo del medio de cultivo y tiempo de incubación utilizados, sino también de la raza del animal evaluado.

6. La utilización del test de reacción acrosómica como predictor de la fertilidad *in vitro* entre diferentes toros tiene una precisión al menos cuestionable cuando se utilizan animales pertenecientes a distintas razas.

7. La calidad morfológica de los ovocitos y los protocolos de maduración utilizados afectan las tasas de anormalidades cromosómicas en los embriones bovinos fertilizados *in vitro*.

8. Los protocolos de maduración ovocitaria utilizados en la producción *in vitro* de embriones bovinos pueden ser optimizados para minimizar la aparición de aberraciones cromosómicas.

General conclusion

The results obtained in the production of bovine embryos by *in vitro* fertilization are influenced by the breed of the animals used and by the percentages of chromosomal abnormalities resulting from the protocols employed.

Conclusions of each chapter.

1. Oocytes belonging to Lidia cattle breed can be obtained, matured and fertilized following standard IVM and IVF protocols.
2. The total number of COC´s and viable oocytes obtained from ovaries derived from Lidia cows are lower than those obtained in other breeds.
3. The use of appropriate hormone supplementation in the maturation media enhances maturation rates, without affecting the fertilization process of these oocytes.
4. The existing doses of frozen semen of Andalusian cattle breeds respond satisfactorily to *in vitro* AR test, allowing their use in future FIV programs.
5. The response and pattern of the *in vitro* AR-induced depend not only on culture medium and incubation time but also on the cattle breed.
6. The accuracy of AR test as predictor of *in vitro* fertility among bulls is questionable when is used among different breeds.
7. Morphological quality of oocytes and maturation protocols affect the chromosomal abnormality rates of the subsequent IVP cattle embryos.

8. Oocyte maturation protocols currently used in *in vitro* cattle embryo production could be optimized to minimize the appearance of chromosomal abnormalities on the subsequent produced embryos.

Producción Científica

Producción Bibliográfica derivada de esta tesis

Demyda, S. E., J. Dorado, M. Hidalgo, D. A. Valls, K. Cano and M. M. Miguel (2010). "Preliminary results of the cytogenetics evaluation of in vitro oocytes maturation in dogs." Chromosome Research **18**(6): 732-733.

Moreno Millan, M., J. O. Quero, L. J. De Luca, M. Hidalgo, J. Dorado and S. E. **Demyda-Peyrás** (2010). "Preliminary results of the use of tumor cells of a dog to condition bovine embryo in vitro culture medium." Reproduction Fertility and Development **22**(1): 301-301.

Moreno Millán, M. and **S. Demyda Peyrás** (2010). "Detection and evaluation of chromosomal aneuploidy as a model for in vitro fertilization processes evaluation (oocytes maturation, sperm capacitation and IVF) in Andalusian autochthonous endangered cattle breeds." Management, economic engineering in agriculture and rural development **10**(1): 8.

Demyda-Peyrás, S., M. Hidalgo, J. Dorado and M. Moreno (2011). "Influence of the sperm fertilization doses on chromosomal abnormality rates of 4-day-old bovine embryos." Reproduction in Domestic Animals **46**: 99-99.

Demyda-Peyras, S., L. J. De Luca and M. M. Millan (2012). "Influence of the sperm selection method and fertilization doses on chromosomal abnormality rates of 4-day-old bovine embryos." Reproduction Fertility and Development **24**(1): 195-195.

Demyda-Peyras, S., J. Dorado, M. Hidalgo, L. De Luca and M. Moreno-Millan (2012). "Preliminar results of the influence of the oocyte maturation media in the chromosomal numeric abnormalities on the in vitro derived early cattle embryos." Chromosome Research **20**(6): 791-791.

Demyda-Peyras, S., J. Dorado, M. Hidalgo, L. De Luca, A. Muñoz-Serrano and M. Moreno-Millan (2012). "In vitro induction of the

acrosome reaction in spermatozoa from endangered Spanish bulls: Effect of breed, culture media and incubation time." Livestock Science **149**(3): 275-281.

Demyda Peyrás, S., J. Dorado, M. Hidalgo, J. Anter, L. De Luca, E. Genero and M. Moreno Millán (2012). "Effects of oocyte quality, maturation environment and incubation time on the number of chromosomal abnormalities in IVF-derived early bovine embryos " Reprod Fert Develop ***In Press***.

Demyda Peyrás, S., M. Hidalgo, J. Dorado, J. Anter, E. Género and M. Moreno Millán (2013). "Short communication: In vitro oocyte maturation and fertilization rates in Spanish the Lidia bovine breed." Spanish Journal of Agricultural Sciences ***In Press***.

Producción Científica producida en colaboración con otros grupos de investigacion realizada durante la tesis

Con el Grupo de Investigación AGR-192 "Reproducción Animal" de la Universidad de Córdoba.

En el laboratorio de Reproducción Animal del Hospital Clínico Veterinario de la Universidad de Córdoba, con los Dres. Jesús Dorado y Manuel Hidalgo.

Dorado, J., M. J. Galvez, M. R. Murabito, **S. Demyda-Peyrás**, L. J. De Luca, M. Moreno and M. Hidalgo (2010). "Effect of eggyolk on the kinematics and acrosome membrane integrity of cooled-rewarmed canine spermatozoa." Reproduction Fertility and Development **22**(1): 204-204.

Hidalgo, M., M. R. Murabito, M. J. Galvez, **S. Demyda-Peyrás**, L. J. De Luca, M. Moreno and J. Dorado (2010). "Assessment of sperm dna fragmentation in canine ejaculates using the sperm-halomax (r) kit: preliminary results." Reproduction Fertility and Development **22**(1): 312-313.

Acha, D., M. Hidalgo, I. Ortiz, **S. Demyda-Peyrás**, M. Herrera, R. Calero, J. Carrasco, B. Diaz, V. Gomez-Arrones and J. Dorado (2011). "Sperm motility patterns in andalusian donkey semen." Reproduction in Domestic Animals **46**: 79-79.

Dorado, J., L. Alcaraz, N. Duarte, J. M. Portero, D. Acha, **S. Demyda-Peyrás**, A. Munoz-Serrano and M. Hidalgo (2011). "Centrifugation on Pure Sperm (R) density-gradient improved quality of spermatozoa from frozen-thawed dog semen." Theriogenology **76**(2): 381-385.

Dorado, J., N. Duarte, L. Alcaraz, J. Portero, D. Acha, I. Ortiz, **S. Demyda-Peyrás**, A. Munoz-Serrano and M. Hidalgo (2011). "Comparison of two

methods to assess the concentration of dog semen." Reproduction in Domestic Animals **46**: 100-100.

Ortiz, I., J. Dorado, D. Acha, L. Ramirez, M. Urbano, M. J. Galvez, L. Alcaraz, J. M. Portero, F. Quesada, C. Gonzalez, **S. Demyda-Peyrás** and M. Hidalgo (2013). "Effect of single-layer centrifugation with equipure (tm) on motility kinematics of frozen-thawed donkey sperm." Reproduction Fertility and Development **25**(1): 183-183.

Quesada, F., J. Dorado, D. Acha, I. Ortiz, M. Urbano, L. Ramirez, M. J. Galvez, L. Alcaraz, J. M. Portero, C. Gonzalez, **S. Demyda-Peyrás** and M. Hidalgo (2013). "Freezing of donkey semen after 24 hours of cool storage: preliminary results." Reproduction Fertility and Development **25**(1): 154-154.

Urbano, M., J. Dorado, I. Ortiz, M. J. Galvez, **S. Demyda-Peyrás**, M. Moreno, L. Alcaraz, L. Ramirez, F. Quesada, C. Gonzalez, J. M. Portero, D. Acha and M. Hidalgo (2013). " Effect of a stressor on canine sperm dna fragmentation using the sperm chromatin dispersion test." Reproduction Fertility and Development **25**(1): 189-190.

Con el Grupo de Investigación AGR-158 MERAGEM "Mejora y de Razas y Genética Molecular" de la Universidad de Córdoba

En el laboratorio de Mutagenesis y Citotoxicidad, con los Dres Jaouad Anter y Ángeles Alonso Moraga.

Anter, J., Z. Fernandez-Bedmar, M. Villatoro-Pulido, **S. Demyda-Peyras**, M. Moreno-Millan, A. Alonso-Moraga, A. Munoz-Serrano and M. D. L. de Castro (2011). "A pilot study on the DNA-protective, cytotoxic, and apoptosis-inducing properties of olive-leaf extracts."

En el laboratorio de Citogenética Animal Aplicada y Molecular con los Dres Antonio Rodero Franganillo y Miguel Moreno Millán

Rodero Franganillo, A., **Demyda-Peyrás S. E.**, Rodero Serrano E., Gonzalez A. and Moreno. Millán, M. (2010). "The 1;29 translocation in Andalusian endangered autochthonous cattle breeds: influence in the general population." Chromosome Research **18**(6): 719-720.

Moreno Millán, M. and **Demyda Peyrás S.** (2011). "Numerical chromosomal abnormalities in three cases of canine neoplasms maintained in culture." Romanian Journal of Comparative Oncology **20**(1): 6.

Demyda-Peyras, S., Rodero Franganillo, A., Rodero Serrano E., Martinez A., Género E. and Moreno-Millan, M. (2012). "Populational study of the t1;29 translocation in Andalusian minority cattle breeds." Chromosome Research **20**(6): 793-793.

Moreno-Millan, M., **Demyda-Peyrás, S.** and Saleno, D. (2012). "Sex chromosomes abnormalities in Purebred Spanish Horses with Reproductive Problems: Case Reports." Bull Univ Ag Sci Vet Med Cluj-Napoca **69**(1): 1-7.

En el laboratorio de Diagnóstico Genético Veterinario con los Dres Alberto Membrillo del Pozo y Antonio Molina Alcalá

Demyda-Peyras, S., Membrillo, A., Bugno-Poniewierska, M., Pawlina, K., Anaya, G. and Moreno-Millán, M. (2013). "The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt." Cytogenet and Genome Research ***In Press***.

Referencias / References

A'Arabi, S.Y., Roussel, J.D., and Chandler, J.E. (1997) Chromosomal analysis of mammalian oocytes matured in vitro with various culture systems. *Theriogenology* **48**(7), 1173-1183

Abraham, M.C., Gustafsson, H., Ruete, A., and Brandt, Y.C. (2012) Breed influences on in vitro development of abattoir-derived bovine oocytes. *Acta Vet Scand* **54**, 36

Albuz, F.K., Sasseville, M., Lane, M., Armstrong, D.T., Thompson, J.G., and Gilchrist, R.B. (2010) Simulated physiological oocyte maturation (SPOM): A novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Human Reproduction* **25**(12), 2999-3011

Alexander, B., Coppola, G., Berardina, D., Rho, G.J., St John, E., Bett, D.H., and King, W.A. (2005) The effect of 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX) on the development and chromosomal complement of sheep parthenogenetic and nuclear transfer embryos. *Mol Reprod Dev*

Ali, A., and Sirard, M.A. (2002) Effect of the absence or presence of various protein supplements on further development of bovine oocytes during in vitro maturation. *Biology of Reproduction* **66**(4), 901-905

Alvarez, R.H., Da Silva, M.V.G.B., De Carvalho, J.B.P., and Binelli, M. (2005) Effects of inbreeding on ovarian responses and embryo production from superovulated Mantiqueira breed cows. *Theriogenology* **64**(8), 1669-1676

Andrabi, S.M.H., and Maxwell, W.M.C. (2007) A review on reproductive biotechnologies for conservation of endangered mammalian species. *Anim Reprod Sci* **99**(3-4), 223-243

Arangasamy, A., and Singh, L.P. (2007) In-vitro capacitation of epididymal spermatozoa with heparin and gelatin binding buffalo seminal plasma proteins. *Indian Journal of Animal Sciences* **77**(7), 549-552

Austin, C.R. (1952) The capacitation of the mammalian sperm. *Nature* **170**(4321), 326

Bar-Anan, R., Osterkorn, K., and Kräusslich, H. (1980) Genetic effects on return intervals in cows. *Liv Prod Sci* **7**(3), 225-233

Barceló-Fimbres, M., Campos-Chillón, L.F., and Seidel, G.E. (2011) In vitro fertilization using non-sexed and sexed bovine sperm: Sperm concentration, sorter pressure, and bull effects. *Reproduction in Domestic Animals* **46**(3), 495-502

Barrett, S.L., and Albertini, D.F. (2010) Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence. *Journal of Assisted Reproduction and Genetics* **27**(1), 29-39

Barros, C.M., Pegorer, M.F., Vasconcelos, J.L.M., Eberhardt, B.G., and Monteiro, F.M. (2006) Importance of sperm genotype (indicus versus taurus) for fertility and embryonic development at elevated temperatures. *Theriogenology* **65**(1), 210-218

Beker, A.R.C.L., Colenbrander, B., and Bevers, M.M. (2002) Effect of 17β-estradiol on the in vitro maturation of bovine oocytes. *Theriogenology* **58**(9), 1663-1673

Bergqvist, A.S., Ballester, J., Johannisson, A., Hernandez, M., Lundeheim, N., and Rodríguez-Martínez, H. (2006) In vitro capacitation of bull spermatozoa by oviductal fluid and its components. *Zygote* **14**(3), 259-273

Bilodeau-Goeseels, S. (2006) Effects of culture media and energy sources on the inhibition of nuclear maturation in bovine oocytes. *Theriogenology* **66**(2), 297-306

Birck, A., Christensen, P., Labouriau, R., Pedersen, J., and Borchersen, S. (2010) In vitro induction of the acrosome reaction in bull sperm and the relationship to field fertility using low-dose inseminations. *Theriogenology* **73**(9), 1180-1191

Birler, S., Pabuççuoğlu, S., Alkan, S., Evecen, M., and Ileri, I.K. (1998) Effects of maturation and fertilization times on pronuclear development of bovine oocytes fertilized in vitro. *In Vitro Fertilize Edilen Siğir Oositlerindeki Pronüklear Gelişim Üzerine Olgunlaştirma ve Fertilizasyon Sürelerinin Etkisi* **22**(1), 1-15

Block, J., Chase Jr, C.C., and Hansen, P.J. (2002) Inheritance of resistance of bovine preimplantation embryos to heat shock: Relative importance of the maternal versus paternal contribution. *Molecular Reproduction and Development* **63**(1), 32-37

Blondin, P., and Sirard, M.A. (1995) Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular Reproduction and Development* **41**(1), 54-62

Boni, R., Cuomo, A., and Tosti, E. (2002) Developmental potential in bovine oocytes is related to cumulus-oocyte complex grade, calcium current activity, and calcium stores. *Biology of Reproduction* **66**(3), 836-842

Brackett, B.G., Bousquet, D., Boice, M.L., Donawick, W.J., Evans, J.F., and Dressel, M.A. (1982) Normal development following in vitro fertilization in the cow. *Biology of Reproduction* **27**(1), 147-158

Brackett, B.G., and Oliphant, G. (1975) Capacitation of rabbit spermatozoa in vitro. *Biology of Reproduction* **12**(2), 260-274

Breininger, E., Cetica, P.D., and Beconi, M.T. (2010) Capacitation inducers act through diverse intracellular mechanisms in cryopreserved bovine sperm. *Theriogenology* **74**(6), 1036-49

Breitbart, H. (2003) Signaling pathways in sperm capacitation and acrosome reaction. *Cellular and molecular biology (Noisy-le-Grand, France)* **49**(3), 321-327

Breuel, K.F., Baker, R.D., Butcher, R.L., Townsend, E.C., Inskeep, E.K., Dailey, R.A., and Lerner, S.P. (1991) Effects of breed, age of donor and dosage of follicle stimulating hormone on the superovulatory response of beef cows. *Theriogenology* **36**(2), 241-255

Burdick, N.C., Randel, R.D., Carroll, J.A., and Welsh, T.H. (2011) Interactions between Temperament, Stress, and Immune Function in Cattle. *International Journal of Zoology* **2011**, 1-9

Burns, B.M., Fordyce, G., and Holroyd, R.G. (2010) A review of factors that impact on the capacity of beef cattle females to conceive, maintain a pregnancy and wean a calf-Implications for reproductive efficiency in northern Australia. *Anim Reprod Sci* **122**(1-2), 1-22

Byrd, W. (1981) In vitro capacitation and the chemically induced acrosome reaction in bovine spermatozoa. *Journal of Experimental Zoology* **215**(1), 35-46

Camargo, L.S.A., Sa, W.F., Ferreira, A.M., and Costa, E.P. (1997a) "In vitro" maturation of bovine oocytes of Nelore breed. *Arquivo Brasileiro De Medicina Veterinaria E Zootecnia* **49**(3), 309-315

Camargo, L.S.A., Sa, W.F., Ferreira, A.M., and Costa, E.P. (1997b) "In vitro" maturation of bovine oocytes of Nelore breed. *Arq Bras Med Vet Zoo* **49**(3), 309-315

Cañón, J., Tupac-Yupanqui, I., García-Atance, M.A., Cortés, O., García, D., Fernández, J., and Dunner, S. (2008) Genetic variation within the Lidia bovine breed. *Anim Genet* **39**(4), 439-445

Carolan, C., Lonergan, P., Monget, P., Monniaux, D., and Mermillod, P. (1996) Effect of follicle size and quality on the ability of follicular fluid to support cytoplasmic maturation of bovine oocytes. *Molecular Reproduction and Development* **43**(4), 477-483

Casali, E., Farruggia, G., and Spisni, A. (1985) Isolation and enzymatic characterization of the plasmalemma from bovine spermatozoa. *Journal of Experimental Zoology* **235**(3), 397-401

Cenariu, M., Pall, E., Cernea, C., and Groza, I. (2012) Evaluation of bovine embryo biopsy techniques according to their ability to preserve embryo viability. *Journal of Biomedicine and Biotechnology* **2012**

Cetica, P.D., Dalvit, G.C., and Beconi, M.T. (1999) Study of evaluation criteria used for in vitro bovine oocyte selection and maturation. *Biocell* **23**(2), 125-133

Collin, S., Sirard, M.A., Dufour, M., and Bailey, J.L. (2000) Sperm calcium levels and chlortetracycline fluorescence patterns are related to the in vivo fertility of cryopreserved bovine semen. *Journal of Andrology* **21**(6), 938-943

Cortés, O., Tupac-Yupanqui, I., García-Atance, M.A., Dunner, S., Fernández, J., and Cañón, J. (2011) Análisis de la variabilidad genética de origen paterno en la raza bovina de lidia. [Paternal genetic variability into the lidia bovine breed]. *Arch Zoot* **60**(231), 417-420

Costa, M.Z., Oliveira, L.Z., Resende, M.V., Lucio, A.C., Perini, A.P., Miguel, M.C.V., and Lima, V.F.M.H. (2010) Induction of the acrosome reaction test to in vitro estimate embryo production in Nelore cattle. *Teste de indução da reação acrossomal na estimativa de produção in vitro de embriões Nelore* **62**(4), 771-777

Coticchio, G., Sereni, E., Serrao, L., Mazzone, S., Iadarola, I., and Borini, A. (2004) What criteria for the definition of oocyte quality? *Annals of the New York Academy of Sciences* **1034**, 132-144

Coy, P., Cánovas, S., Mondéjar, I., Saavedra, M.D., Romar, R., Grullón, L., Matás, C., and Avilés, M. (2008) Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proceedings of the National Academy of Sciences of the United States of America* **105**(41), 15809-15814

Coy, P., Gadea, J., Romar, R., Matás, C., and García, E. (2002) Effect of in vitro fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and in vitro development in pigs. *Reproduction* **124**(2), 279-288

Cross, N.L. (1998) Role of cholesterol in sperm capacitation. *Biology of Reproduction* **59**(1), 7-11

Cross, N.L., and Watson, S.K. (1994) Assessing acrosomal status of bovine sperm using fluoresceinated lectins. *Theriogenology* **42**(1), 89-98

Cushman, R.A., Allan, M.F., Snowder, G.D., Thallman, R.M., and Echternkamp, S.E. (2005) Evaluation of ovulation rate and ovarian phenotype in puberal heifers from a cattle population selected for increased ovulation rate. *J Anim Sci* **83**(8), 1839-1844

Cheng, F.P., Fazeli, A., Voorhout, W.F., Marks, A., Bevers, M.M., and Colenbrander, B. (1996) Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome eaction in stallion spermatozoa. *Journal of Andrology* **17**(6), 674-682

Choi, Y.H., Carnevale, E.M., Seidel Jr, G.E., and Squires, E.L. (2001) Effects of gonadotropins on bovine oocytes matured in TCM-199. *Theriogenology* **56**(4), 661-670

Christopikou, D., Karamalegos, C., Doriza, S., Argyrou, M., Sisi, P., Davies, S., and Mastrominas, M. (2010) Spindle and chromosome configurations of human oocytes matured in vitro in two different culture media. *Reproductive BioMedicine Online* **20**(5), 639-648

Dale, B., and Defelice, L. (2011) Polyspermy prevention: Facts and artifacts? *Journal of Assisted Reproduction and Genetics* **28**(3), 199-207

Dapino, D.G., Marini, P.E., and Cabada, M.O. (2006) Effect of heparin on in vitro capacitation of boar sperm. *Biological Research* **39**(4), 631-639

de Armas, R., Solano, R., Riego, E., Pupo, C.A., Aguilar, A., Ramos, B., Aguirre, A., de la Fuente, J., and Castro, F.O. (1994) Use of F1 progeny of Holstein × Zebu cross cattle as oocyte donors for in vitro embryo production and gene microinjection. *Theriogenology* **42**(6), 977-985

de Lima, V.F.M.H., Moreira-Filho, C.A., de Lucio, A.C., and Resende, M.V. (2011) Sex selection in bovine spermatozoa by using percoll discontinuos density gradient centrifugation. *Sexagem de espermatozoides bovinos por centrifugação em gradiente descontínuo de densidade de percoll* **40**(8), 1680-1685

Demyda-Peyras, S., Dorado, J., Hidalgo, M., Anter, J., De Luca, L., Genero, E., and Moreno-Millan, M. (2012) Effects of oocyte quality, incubation time and maturation environment on the number of chromosomal abnormalities in IVF-derived early bovine embryos. *Reprod Fertil Dev* **25**(7)

Demyda Peyrás , S., Membrillo, A., Bugno Poniewierska, M., Pawlina, K., Anaya, G., and Moreno Millán, M. (2013) The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: The case of a sexual chimerismin a Spanish Purebred foal. *Cytogenet and Genome Research* ***In Press***

DiBerardino, D., Hayes, H., Fries, R., and Long, S. (1990) International system for cytogenetic nomenclature of domestic animals (1989). The second International Conference on Standardization of Domestic Animal Karyotypes. *Cytogenetics and Cell Genetics* **53**(2-3), 65-79

Dieleman, S.J., Hendriksen, P.J.M., Viuff, D., Thomsen, P.D., Hyttel, P., Knijn, H.M., Wrenzycki, C., Kruip, T.A.M., Niemann, H., Gadella, B.M., Bevers, M.M., and Vos, P.L.A.M. (2002) Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology* **57**(1), 5-20

Dode, M.A.N., Rodovalho, N.C., Ueno, V.G., and Alves, R.G.O. (2001) Oocyte recovering from Bos Indicus Females. *Arch Zoot* **50**(1), 415-418

Dode, M.A.N., Rodovalho, N.C., Ueno, V.G., and Fernandes, C.E. (2002) The effect of sperm preparation and co-incubation time on in vitro fertilization of bos indicus oocytes. *Anim Reprod Sci* **69**(1-2), 15-23

Domínguez, M.M. (1995) Effects of body condition, reproductive status and breed on follicular population and oocyte quality in cows. *Theriogenology* **43**(8), 1405-1418

Dunn, H.O., McEntee, K., and Hansel, W. (1970) Diploid-triploid chimerism in a bovine true hermaphrodite. *Cytogenetics* **9**(4), 245-259

Duszewska, A.M., Raçpala, L., Trzeciak, P., Daçbrowski, S., and Piliszek, A. (2012) Obtaining farm animal embryos in vitro. *Journal of Animal and Feed Sciences* **21**(2), 217-233

Eberhardt, B.G., Satrapa, R.A., Capinzaiki, C.R.L., Trinca, L.A., and Barros, C.M. (2009) Influence of the breed of bull (Bos taurus indicus vs. Bos taurus taurus) and the breed of cow (Bos taurus indicus, Bos taurus taurus and crossbred) on the resistance of bovine embryos to heat. *Animal Reproduction Science* **114**(1-3), 54-61

Ebner, T., Moser, M., Sommergruber, M., and Tews, G. (2003) Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: A review. *Human Reproduction Update* **9**(3), 251-262

Eddy, E.M., Clark, J.M., Gong, D., and Fenderson, B.A. (1981) Origin and migration of primordial germ cells in mammals. *Gamete Research* **4**(4), 333-362

Edwards, J.L., Saxton, A.M., Lawrence, J.L., Payton, R.R., and Dunlap, J.R. (2005) Exposure to a physiologically relevant elevated temperature hastens in vitro maturation in bovine oocytes. *Journal of Dairy Science* **88**(12), 4326-4333

Egerszegi, I., Torner, H., Rátky, J., and Brüssow, K.P. (2001) Follicular development and preovulatory oocyte maturation in Hungarian Mangalica and Landrace gilts. *Arch Tierzucht* **44**(4), 413-419

Eichenlaub-Ritter, U., Wieczorek, M., Lüke, S., and Seidel, T. (2011) Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. *Mitochondrion* **11**(5), 783-796

England, G., and Russo, M. (2006) Conception problems in the bitch. *In Practice* **28**(10), 588-597

Evans, A.C.O., Mossa, F., Fair, T., Lonergan, P., Smith, G.W., Jimenez-Krassel, F., Folger, J.K., Ireland, J.L.H., and Ireland, J.J. (2010) Variation in the number of ovarian follicles in cattle: Possible causes and consequences. *Acta Sci Veter* **38**(SUPPL. 2), s537-s543

Ferreira, R.M., Ayres, H., Ferraz, M.L., Araújo, A.B., Chiaratti, M.R., Sá Filho, M.F., Calomeni, G., Rodrigues, C.A., Watanabe, Y.F., Vireque, A.A., and Baruselli, P.S. (2009) Ovum pickup and in vitro embryo production during the summer: differences between Holstein heifers, High-producing Holstein cows in peak lactation, and repeat-breeder Holstein cows. *Reproduction, Fertility and Development* **22**(1), 290-290

Fields, M.J., Burns, W.C., and Warnick, A.C. (1979) Age, season and breed effects on testicular volume and semen traits in young beef bulls. *Journal of animal science* **48**(6), 1299-1304

Fischer, A.E., Bernal, D.P., Gutierrez-Robayo, C., and Rutledge, J.J. (2000) Estimates of heterosis for in vitro embryo production using reciprocal crosses in cattle. *Theriogenology* **54**(9), 1433-1442

Flaherty, S., Payne, D., Swann, N., and Matthews, C. (1995) Assessment of fertilization failure and abnormal fertilization after intracytoplasmic sperm injection (ICSI). *Reprod Fert Develop* **7**(2), 197-210

Frumkin, T., Malcov, M., Yaron, Y., and Ben-Yosef, D. (2008) Elucidating the origin of chromosomal aberrations in IVF embryos by preimplantation genetic analysis. *Molecular and Cellular Endocrinology* **282**(1–2), 112-119

Fukuda, Y., and Enari, N. Developmental ability of in vitro matured-in vitro fertilized bovine embryos derived from oocytes with homogeneous or heterogenous ooplasm. In '7th World Conference on Animal Production', 1993, pp. 276-277

Fukui, Y., Fukushima, M., Terawaki, Y., and Ono, H. (1982) Effect of gonadotropins, steroids and culture media on bovine oocyte maturation in vitro. *Theriogenology* **18**(2), 161-175

Fukui, Y., and Ono, H. (1989) Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. *Journal of Reproduction and Fertility* **86**(2), 501-506

Fukui, Y., Sonoyama, T., Mochizuki, H., and Ono, H. (1990) Effects of heparin dosage and sperm capacitation time on in vitro fertilization and cleavage of bovine oocytes matured in vitro. *Theriogenology* **34**(3), 579-591

Funahashi, H. (2003) Polyspermic penetration in porcine IVM-IVF systems. *Reproduction, Fertility and Development* **15**(3-4), 167-177

Gandini, G., Pizzi, F., Stella, A., and Boettcher, P.J. (2007) The costs of breed reconstruction from cryopreserved material in mammalian livestock species. *Genetics Selection Evolution* **39**(4), 465-479

García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J.J., Ramón, M., Fernández-Santos, M.R., Esteso, M.C., Pérez-Guzmán, M.D., and Soler, A.J. (2009) Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection. *Theriogenology* **72**(2), 160-168

Garcia-Herreros, M., Carter, T.F., Villagmez, D.A.F., MacAulay, A.D., Rath, D., King, W.A., and Lonergan, P. (2010) Incidence of chromosomal abnormalities in bovine blastocysts derived from unsorted and sex-sorted spermatozoa. *Reproduction, Fertility and Development* **22**(8), 1272-1278

Getz, I., Karadjole, M., Samardzija, M., Macesic, N., Makek, Z., Karadjole, T., Dobranic, T., Bacic, G., and Vince, S. (2008) Effect of Breed (Simmental and Holstein-Frisian) of Donar Cows on Ovum Pick-up/In Vitro Fertilization Results after Repeated Superovulation with FSH-P. *Reproduction in Domestic Animals* **43**, 79-79

Ghoraeian, P., Mozdarani, H., Aleyasin, A., and Alizadeh-Nili, H. (2013) Frequency of sex chromosomal disomy in spermatozoa of normal and oligozoospermic Iranian patients and its effects on fertilisation and implantation rates after ICSI. *Andrologia* **45**(1), 46-55

Gianaroli, L., Magli, M.C., Ferraretti, A.P., Lappi, M., Borghi, E., and Ermini, B. (2007) Oocyte euploidy, pronuclear zygote morphology and embryo chromosomal complement. *Human Reproduction* **22**(1), 241-249

Giritharan, G., Ramakrishnappa, N., Balendran, A., Cheng, K.M., and Rajamahendran, R. (2005) Development of in vitro tests to predict fertility of bulls. *Canadian Journal of Animal Science* **85**(1), 47-52

Gliedt, D.W., Rosenkrans Jr, C.F., Rorie, R.W., and Rakes, J.M. (1996) Effects of Oocyte Maturation Length, Sperm Capacitation Time, and Heparin on Bovine Embryo Development. *Journal of Dairy Science* **79**(4), 532-535

Gustavsson, I., Hageltorn, M., and Zech, L. (1976) Identification of the 1/29-translocation in the Swedish Red and White (SRB) cattle breed by utilization of new staining techniques. *Hereditas* **82**(2), 260-2

Guyader-Joly, C., Moulin, B., Mariller, F., Curin, V., Ponchon, S., Gonzalez, C., Humblot, P., and Ponsart, C. (2009) Biological factors affecting oocyte collection and embryos production in a commercial ovum pickup-ivp system in holstein and montbeliard breeds. *Reproduction, Fertility and Development* **22**(1), 378-379

Hansen, P.J. (2006) Realizing the promise of IVF in cattle - An overview. *Theriogenology* **65**(1), 119-125

Harton, G.L., and Tempest, H.G. (2012) Chromosomal disorders and male infertility. *Asian Journal of Andrology* **14**(1), 32-39

Havlicek, V., Lopatarova, M., Cech, S., Dolezel, R., Huber, T., Pavlok, A., Brem, G., and Besenfelder, U. (2005) In vivo culture of bovine embryos and quality assessment of in vivo vs. in vitro produced embryos. *Veterinarni Medicina* **50**(4), 149-157

Hawk, H.W., and Wall, R.J. (1994) Improved yields of bovine blastocysts from in vitro-produced oocytes. II. Media and co-culture cells. *Theriogenology* **41**(8), 1585-1594

Hazeleger, N.L., Hill, D.J., Stubbing, R.B., and Walton, J.S. (1995) Relationship of morphology and follicular fluid environment of bovine oocytes to their developmental potential in vitro. *Theriogenology* **43**(2), 509-522

Hernández-Cerón, J., Chase Jr, C.C., and Hansen, P.J. (2004) Differences in heat tolerance between preimplantation embryos from Brahman, Romosinuano, and Angus breeds. *Journal of Dairy Science* **87**(1), 53-58

Herrera, J., Fierro, R., Zayas, H., Conejo, J., Jiménez, I., García, A., and Betancourt, M. (2002) Acrosome reaction in fertile and subfertile boar sperm. *Systems Biology in Reproductive Medicine* **48**(2), 133-139

Hinrichs, K. (2013) Assisted reproduction techniques in the horse. *Reproduction, Fertility and Development* **25**(1), 80-93

Hochi, S., Korosue, K., Choi, Y.H., and Oguri, N. (1996) In vitro capacitation of stallion spermatozoa assessed by the lysophosphatidylcholine-induced acrosome reaction and the penetration rate into in vitro-matured, zona-free mare oocytes. *Journal of Equine Veterinary Science* **16**(6), 244-248

Hoflack, G., Van Soom, A., Maes, D., de Kruif, A., Opsomer, G., and Duchateau, L. (2006) Breeding soundness and libido examination of Belgian Blue and Holstein Friesian artificial insemination bulls in Belgium and The Netherlands. *Theriogenology* **66**(2), 207-216

Hornak, M., Jeseta, M., Musilova, P., Pavlok, A., Kubelka, M., Motlik, J., Rubes, J., and Anger, M. (2011) Frequency of aneuploidy related to age in porcine oocytes. *PLoS ONE* **6**(4)

Hornak, M., Oracova, E., Hulinska, P., Urbankova, L., and Rubes, J. (2012) Aneuploidy detection in pigs using comparative genomic hybridization: From the oocytes to blastocysts. *PLoS ONE* **7**(1)

Humblot, P., Le Bourhis, D., Fritz, S., Colleau, J.J., Gonzalez, C., Guyader Joly, C., Malafosse, A., Heyman, Y., Amigues, Y., Tissier, M., and Ponsart, C. (2010) Reproductive technologies and genomic selection in cattle. *Veterinary Medicine International* **2010**

Hyslop, L., Prathalingam, N., Nowak, L., Fenwick, J., Harbottle, S., Byerley, S., Rhodes, J., Watson, B., Henderson, R., Murdoch, A., and Herbert, M. (2012) A novel isolator-based system promotes viability of human embryos during laboratory processing. *PLoS ONE* **7**(2)

Hyttel, P., Fair, T., Callesen, H., and Greve, T. (1997) Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* **47**(1), 23-32

IETS (1998-2012) Data Retrieval Comitte Report. In '.' (IETS)

Ijaz, A., and Hunter, A.G. (1989) Evaluation of calcium-free Tyrode's sperm capacitation medium for use in bovine in vitro fertilization. *Journal of dairy science* **72**(12), 3280-3285

Jacobs, P.A., Szulman, A.E., Funkhouser, J., Matsuura, J.S., and Wilson, C.C. (1982) Human triploidy: Relationship between parental origin of the additional haploid complement and development of partial hydatidiform mole. *Annals of Human Genetics* **46**(3), 223-231

Januskauskas, A., Gil, J., Söderquist, L., and Rodriguez-Martinez, H. (2000) Relationship between sperm response to glycosaminoglycans in vitro and non-return rates of Swedish dairy AI bulls. *Reproduction in Domestic Animals* **35**(5), 207-212

Januskauskas, A., Söderquist, L., Håård, M.G., Håård, M.C., Lundeheim, N., and Rodriguez-Martinez, H. (1996) Influence of Sperm Number Per Straw on the Post-Thaw Sperm Viability and Fertility of Swedish Red and White A.I. Bulls. *Acta veterinaria Scandinavica* **37**(4), 461-470

Jimenez, J.M., Criado, M., and Molina, A. (2007) Las Razas Bovinas de Fomento Andaluzas: Retinta y Lidia. In 'Las Razas Ganaderas de Andalucía. Vol. II.' (Eds. A Rodero Franganillo and E Rodero Serrano) pp. 9-52. (Junta de de Andalucía: Sevilla (Spain))

Kafi, M., McGowan, M.R., and Kirkland, P.D. (2002) In vitro maturation and fertilization of bovine oocytes and in vitro culture of presumptive zygotes in the presence of bovine pestivirus. *Anim Reprod Sci* **71**(3-4), 169-179

Kato, Y., and Nagao, Y. (2009) Effect of PVP on sperm capacitation status and embryonic development in cattle. *Theriogenology* **72**(5), 624-35

Katska-Ksiazkiewicz, L., Lechniak-Cieślak, D., Korwin-Kossakowska, A., Alm, H., Ryńska, B., Warzych, E., Sosnowski, J., and Sender, G. (2006) Genetical and biotechnological methods of utilization of female reproductive potential in mammals. *Reprod Biology* **6 Suppl 1**, 21-36

Katska, L., and Smorag, Z. (1996) Bull effect on in vitro embryo production in cattle. *Journal of Physiology and Pharmacology* **47**(2 SUPPL. 1), 71-78

Kawarsky, S.J., Basrur, P.K., Stubbings, R.B., Hansen, P.J., and King, W.A. (1996) Chromosomal abnormalities in bovine embryos and their influence on development. *Biology of Reproduction* **54**(1), 53-59

Keskintepe, L., and Brackett, B.G. (1996) In vitro developmental competence of in vitro-matured bovine oocytes fertilized and cultured in completely defined media. *Biology of Reproduction* **55**(2), 333-339

Kharche, S.D., Goel, A.K., Jindal, S.K., Yadav, E.N., Yadav, P., Sinha, R., and Sinha, N.K. (2009) Effect of serum albumin supplementation on in vitro capacitation and fertilization of caprine oocytes. *Small Ruminant Research* **81**(2-3), 85-89

Khatib, H., Monson, R.L., Huang, W., Khatib, R., Schutzkus, V., Khateeb, H., and Parrish, J.J. (2010) Short communication: Validation of in vitro fertility genes in a Holstein bull population. *J Dairy Sci* **93**(5), 2244-2249

Khatir, H., Lonergan, P., Touzé, J.L., and Mermillod, P. (1998) The characterization of bovine embryos obtained from prepubertal calf oocytes and their viability after non surgical embryo transfer. *Theriogenology* **50**(8), 1201-1210

King, W.A. (1990) Chromosome abnormalities and pregnancy failure in domestic animals. *Adv Vet Sci Comp Med* **34**, 229-250

King, W.A. (2008) Chromosome variation in the embryos of domestic animals. *Cytogenetic and Genome Research* **120**(1-2), 81-90

King, W.A., Coppola, G., Alexander, B., Mastromonaco, G., Perrault, S., Nino-Soto, M.I., Pinton, A., Joudrey, E.M., and Betts, D.H. (2006) The impact of chromosomal alteration on embryo development. *Theriogenology* **65**(1), 166-77

Knijn, H.M., Wrenzycki, C., Hendriksen, P.J.M., Vos, P.L.A.M., Herrmann, D., van der Weijden, G.C., Niemann, H., and Dieleman, S.J. (2002) Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. *Reproduction* **124**(3), 365-375

Krisher, R.L. (2004) The effect of oocyte quality on development. *Journal of animal science.* **82 E-Suppl**, E14-23

Kuroda, K., Fukushima, M., and Harayama, H. (2007) Premature capacitation of frozen-thawed spermatozoa from subfertile Japanese Black cattle. *Journal of Reproduction and Development* **53**(5), 1079-1086

Lacadena, J.R. (1996) 'Citogenética.' Primera edn. (Madrid, España) 996

Lacham-Kaplan, O., and Trounson, A. (2008) Reduced developmental competence of immature, in-vitro matured and postovulatory aged mouse oocytes following IVF and ICSI. *Reproductive Biology and Endocrinology* **6**

Lechniak, D., and Switonski, M. (1998) Aneuploidy in bovine oocytes matured in vitro. *Chromosome Res* **6**(6), 504-6

Lechniak, D., Świtoński, M., and Sosnowski, M. (1996) The incidence of bovine diploid oocytes matured in vitro. *Theriogenology* **46**(2), 267-277

Lechniak, D., Szczepankiewicz, D., Kauss, D., Szulc, J., and Szydłowski, M. (2005) IVM media, oocyte diameter and donor genotype at RYR1 locus in relation to the incidence of porcine diploid oocytes after maturation in vitro. *Theriogenology* **64**(1), 202-212

Lee, S.R., Kim, B.S., Kim, J.W., Kim, M.O., Kim, S.H., Yoo, D.H., Shin, M.J., Park, Y.S., Lee, S., Park, Y.B., Ha, J.H., and Ryoo, Z.Y. (2007) In vitro maturation, in vitro fertilization and embryonic development of canine oocytes. *Zygote* **15**(4), 347-353

Lessard, C., Siqueira, L.G., D'Amours, O., Sullivan, R., Leclerc, P., and Palmer, C. (2011) Infertility in a beef bull due to a failure in the capacitation process. *Theriogenology* **76**(5), 891-899

Li, G.P., Liu, Y., White, K.L., and Bunch, T.D. (2005) Cytogenetic analysis of diploidy in cloned bovine embryos using an improved air-dry karyotyping method. *Theriogenology* **63**(9), 2434-2444

Lim, J.M., Kim, J.H., Okuda, K., and Niwa, K. (1994) The importance of NaCl concentration in a chemically defined medium for the development of bovine oocytes matured and fertilized in vitro. *Theriogenology* **42**(3), 421-432

Liu, L., and Keefe, D.L. (2002) Ageing-associated aberration in meiosis of occytes from senescence-accelerated mice. *Human Reproduction* **17**(10), 2678-2685

Liu, S., Feng, H.L., Marchesi, D., Chen, Z.J., and Hershlag, A. (2011) Dose-dependent effects of gonadotropin on oocyte developmental competence and apoptosis. *Reproduction, Fertility and Development* **23**(8), 990-996

Lonergan, P. (2007) State-of-the-art embryo technologies in cattle. *Society of Reproduction and Fertility supplement* **64**, 315-325

Lonergan, P., Pedersen, H.G., Rizos, D., Greve, T., Thomsen, P.D., Fair, T., Evans, A., and Boland, M.P. (2004) Effect of the post-fertilization culture environment on the incidence of chromosome aberrations in bovine blastocysts. *Biology of Reproduction* **71**(4), 1096-1100

Long, C.R., Damiani, P., Pinto-Correia, C., MacLean, R.A., Duby, R.T., and Robl, J.M. (1994) Morphology and subsequent development in culture of bovine oocytes matured in vitro under various conditions of fertilization. *Journal of Reproduction and Fertility* **102**(2), 361-369

Lu, K.H., Gordon, I., Gallagher, M., and McGovern, H. (1987) Pregnancy established in cattle by transfer of embryos derived from in vitro fertilisation of oocytes matured in vitro. *Veterinary Record* **121**(11), 259-260

Lukoseviciute, K., Zilinskas, H., and Januskauskas, A. (2004) Effect of exogenous progesterone on post-thaw capacitation and acrosome reaction of bovine spermatozoa. *Reproduction in Domestic Animals* **39**(3), 154-161

MacFeely, R.A., and Rajakoski, E. Chromosome studies on early embryos of the cow. In 'Proc Int Congr Anim Reprod Artif Insem ', 1968, pp. 905-907

Machatkova, M., Horakova, J., Hulinska, P., Reckova, Z., and Hanzalova, K. (2008a) Early oocyte penetration can predict the efficiency of bovine embryo production in vitro. *Zygote* **16**(3), 203-209

Machatkova, M., Horakova, J., Rybar, R., Hanzalova, K., and Rubes, J. (2005) Embryos produced in vitro from bulls carrying 16;20 and 1;29 Robertsonian translocations: Efficiency and kinetics of oocyte fertilization and embryo development. *Zygote* **13**(2), 97-101

Machatkova, M., Hulinska, P., Reckova, Z., Hanzalova, K., Spanihelova, J., and Pospisil, R. (2008b) In vitro production of embryos from high performance cows and the development of frozen-thawed embryos after transfer: A field study. *Vet Med-Czech* **53**(7), 358-364

Mapletoft, R.J., and Hasler, J.F. (2005) Assisted reproductive technologies in cattle: A review. *OIE Revue Scientifique et Technique* **24**(1), 393-403

Máximo, D.M., Martins da Silva, I.G., Mondadori, R.G., Neves, J.P., and Lucci, C.M. (2012) Ultrastructural characteristics of sheep oocytes during in vitro maturation (IVM). *Small Ruminant Research* **105**(1-3), 210-215

McFadden, D.E., and Friedman, J.M. (1997) Chromosome abnormalities in human beings. *Mutat Res* **396**(1-2), 129-40

McLaughlin, M., and Telfer, E.E. (2010) Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. *Reproduction* **139**(6), 971-978

Meczekalski, B. (2009) Oocyte-specific genes: Role in fertility and infertility. *J Endocrinol Invest* **32**(5), 474-481

Meinecke, B., Drögemüller, C., Kuiper, H., Bürstel, D., Wohlsein, P., Ebeling, S., Wehrend, A., and Meinecke-Tillmann, S. (2007) A diploid-triploid (60,XX/90,XXY) intersex in a Holstein heifer. *Sexual Development* **1**(1), 59-65

Menon, A.G., Barkema, H.W., Wilde, R., Kastelic, J.P., and Thundathil, J.C. (2011) Associations between sperm abnormalities, breed, age, and scrotal circumference in beef bulls. *Canadian Journal of Veterinary Research* **75**(4), 241-247

Mermillod, P., Schmaltz, B., Perreau, C., Tsikis, G., Martinot, E., Cordova, A., and Locatelli, Y. (2010) Early development of ruminant embryos, autonomous process or the result of a positive dialog with surrounding maternal tissues? *Acta Scientiae Veterinariae* **38**(SUPPL. 2), s521-s533

Merton, J.S., Ask, B., Onkundi, D.C., Mullaart, E., Colenbrander, B., and Nielen, M. (2009) Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-in vitro production embryo-production program. *Theriogenology* **72**(7), 885-893

Meyers, S.A., Liu, I.K.M., Overstreet, J.W., Vadas, S., and Drobnis, E.Z. (1996) Zona pellucida binding and zona-induced acrosome reactions in horse spermatozoa: Comparisons between fertile and subfertile stallions. *Theriogenology* **46**(7), 1277-1288

Miller, D.J., Winer, M.A., and Ax, R.L. (1990) Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. *Biology of Reproduction* **42**(5-6), 899-915

Mirkena, T., Duguma, G., Haile, A., Tibbo, M., Okeyo, A.M., Wurzinger, M., and Sölkner, J. (2010) Genetics of adaptation in domestic farm animals: A review. *Livestock Science* **132**(1–3), 1-12

Molnarova, Z., Machatkova, M., Machal, L., Horakova, J., and Hanzalova, K. (2006) A potential relationship between the acrosome response characteristics of bovine spermatozoa and their in vitro fertilizing ability. *Zygote* **14**(1), 63-69

Moosani, N., Pattinson, H.A., Carter, M.D., Cox, D.M., Rademaker, A.W., and Martin, R.H. (1995) Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence in situ hybridization. *Fertility and Sterility* **64**(4), 811-817

Moreno Millán, M., and Demyda Peyrás , S. (2010) Detection and evaluation of chromosomal aneuploidy as a model for in vitro fertilization processes evaluation (oocytes maturation, sperm capacitation and IVF) in Andalusian autochthonous endangered cattle breeds. *Management, economic engineering in agriculture and rural development* **10**(1), 8

Morgan, J.F., Campbell, M.E., and Morton, H.J. (1955) The nutrition of animal tissues cultivated in vitro. I. A survey of natural materials as supplements to synthetic medium 199. *Journal of the National Cancer Institute* **16**(2), 557-567

Mukhopadhyay, C.S., Gupta, A.K., Yadav, B.R., Chauhan, I.S., Gupta, A., Mohanty, T.K., and Raina, V.S. (2011) Effect of cryopreservation on sperm chromatin integrity and fertilizing potential in bovine semen. *Livestock Science* **136**(2-3), 114-121

Mukhopadhyay, C.S., Verma, A., Joshi, B.K., Singh, A., Chakravarty, A.K., and Dubey, P.P. (2008) In Vitro acrosome reaction: Its pertinence in assessing fertility of cryopreserved semen. *Indian Journal of Animal Research* **42**(3), 201-204

Munné, S., Chen, S., Collis, P., Garrisi, J., Zheng, X., Cekleniak, N., Lenzi, M., Hughes, P., Fischer, J., Garrisi, M., Tomkin, G., and Cohen, J. (2007) Maternal age, morphology, development and chromosome abnormalities in over 6000 cleavage-stage embryos. *Reproductive BioMedicine Online* **14**(5)

Munné, S., Márquez, C., Reing, A., Garrisi, J., and Alikani, M. (1998) Chromosome abnormalities in embryos obtained after conventional in vitro fertilization and intracytoplasmic sperm injection. *Fertility and Sterility* **69**(5), 904-908

Murray, J.D., Moran, C., Boland, M.P., Doff, A.M., and Nancarrow, C.D. (1985) Cytogenetic analysis of 34 early stage bovine embryos from superovulated Hereford donors. *Canadian Journal of Genetics and Cytology* **27**(4), 483-486

Nagai, T. (2001) The improvement of in vitro maturation systems for bovine and porcine oocytes. *Theriogenology* **55**(6), 1291-1301

Nagano, M., Katagiri, S., and Takahashi, Y. (2006) Relationship between bovine oocyte morphology and in vitro developmental potential. *Zygote* **14**(1), 53-61

Nagaoka, S.I., Hassold, T.J., and Hunt, P.A. (2012) Human aneuploidy: Mechanisms and new insights into an age-old problem. *Nature Reviews Genetics* **13**(7), 493-504

Nicodemo, D., Pauciullo, A., Castello, A., Roldan, E., Gomendio, M., Cosenza, G., Peretti, V., Perucatti, A., Di Meo, G.P., Ramunno, L., Iannuzzi, L., Rubes, J., and Di Berardino, D. (2009) X-Y sperm aneuploidy in 2 cattle (bos taurus) breeds as determined by dual color fluorescent in situ hybridization (fish). *Cytogenetic and Genome Research* **126**(1-2), 217-225

Nicodemo, D., Pauciullo, A., Cosenza, G., Peretti, V., Perucatti, A., Di Meo, G.P., Ramunno, L., Iannuzzi, L., Rubes, J., and Di Berardino, D. (2010) Frequency of aneuploidy in in vitro-matured MII oocytes and corresponding first polar bodies in two dairy cattle (Bos taurus) breeds as determined by dual-color fluorescent in situ hybridization. *Theriogenology* **73**(4), 523-529

Niemann, H., and Kues, W.A. (2003) Application of transgenesis in livestock for agriculture and biomedicine. *Animal Reproduction Science* **79**(3-4), 291-317

Niwa, K. (1993) Effectiveness of in vitro maturation and in vitro fertilization techniques in pigs. *Journal of reproduction and fertility. Supplement* **48**, 49-59

Niwa, K., and Ohgoda, O. (1988) Synergistic effect of caffeine and heparin on in-vitro fertilization of cattle oocytes matured in culture. *Theriogenology* **30**(4), 733-741

Nunes Dode, M.A., Rodovalho, N.C., Ueno, V.G., and De Oliveira Alves, R.G. (2000) Effect of follicle size in nuclear and cytoplasmic maturation of oocytes from zebu cows. *Efeito do tamanho do folículo na maturação nuclear e citoplasmática de ovócitos de fêmeas zebuínas* **35**(1), 207-214

Ocana-Quero, J.M., Pinedo-Merlin, M., and Moreno-Millan, M. (1999a) Cytogenetic study of in vitro-derived bovine embryos. *Vet J* **158**(3), 228-33

Ocana-Quero, J.M., Pinedo-Merlin, M., and Moreno-Millan, M. (1999b) Influence of follicle size, medium, temperature and time on the incidence of diploid bovine oocytes matured in vitro. *Theriogenology* **51**(3), 667-72

Ocana Quero, J.M., Gomez Villamandos, R.J., Moreno Millan, M., and Santisteban Valenzuela, J.M. (1995) The effect of helium-neon laser irradiation on in vitro maturation and fertilization of immature bovine oocytes. *Laser Med Sci* **10**(2), 113-119

Ocana Quero, J.M., Moreno Millan, M., Valera Cordoba, M., and Rodero Franganillo, A. (1994) The influence of different types of media supplement on the meiotic maturation of bovine oocytes in vitro. *Theriogenology* **41**(2), 405-411

Olsen, H.G., Hayes, B.J., Kent, M.P., Nome, T., Svendsen, M., Larsgard, A.G., and Lien, S. (2011) Genome-wide association mapping in Norwegian Red cattle identifies quantitative trait loci for fertility and milk production on BTA12. *Anim Genet* **42**(5), 466-474

Ott, M., Schernthaner, W., Sinowatz, F., and Wolf, E. (2002) Effects of bovine serum albumin and estrous cow serum on development and ultrastructure of in vitro-produced porcine embryos. *Anatomia, Histologia, Embryologia* **31**(3), 151-157

Palasz, A.T., Rodriguez-Martinez, H., Beltran-Breña, P., Perez-Garnelo, S., Martinez, M.F., Gutierrez-Adan, A., and De La Fuente, J. (2006) Effects of hyaluronan, BSA, and serum on bovine embryo in vitro development, ultrastructure, and gene expression patterns. *Molecular Reproduction and Development* **73**(12), 1503-1511

Palma, G.A., and Sinowatz, F. (2004) Male and female effects on the in vitro production of bovine embryos. *Anat Histol Embryol* **33**(5), 257-62

Pan, H., Ma, P., Zhu, W., and Schultz, R.M. (2008) Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Developmental Biology* **316**(2), 397-407

Panetto, J.C.C., Gutiérrez, J.P., Ferraz, J.B.S., Cunha, D.G., and Golden, B.L. (2010) Assessment of inbreeding depression in a Guzerat dairy herd: Effects of individual increase in inbreeding coefficients on production and reproduction. *Journal of Dairy Science* **93**(10), 4902-4912

Park, Y.S., Kim, S.S., Kim, J.M., Park, H.D., and Byun, M.D. (2005) The effects of duration of in vitro maturation of bovine oocytes on subsequent development, quality and transfer of embryos. *Theriogenology* **64**(1), 123-134

Parrish, J.J., Krogenaes, A., and Susko-Parrish, J.L. (1995) Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. *Theriogenology* **44**(6), 859-869

Parrish, J.J., Susko-Parrish, J., Winer, M.A., and First, N.L. (1988a) Capacitation of bovine sperm by heparin. *Biology of Reproduction* **38**(5), 1171-1180

Parrish, J.J., Susko-Parrish, J., Winer, M.A., and First, N.L. (1988b) Capacitation of bovine sperm by heparin. *Biol Reprod* **38**(5), 1171-1180

Parrish, J.J., Susko-Parrish, J.L., and Graham, J.K. (1999) In vitro capacitation of bovine spermatozoa: Role of intracellular calcium. *Theriogenology* **51**(2), 461-472

Parrish, J.J., Susko-Parrish, J.L., Handrow, R.R., Ax, R.L., and First, N.L. (1989a) Effect of sulfated glycoconjugates on capacitation and the acrosome reaction of bovine and hamster spermatozoa. *Gamete Research* **24**(4), 403-413

Parrish, J.J., Susko-Parrish, J.L., Handrow, R.R., Sims, M.M., and First, N.L. (1989b) Capacitation of bovine spermatozoa by oviduct fluid. *Biology of Reproduction* **40**(5), 1020-1025

Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H., and First, N.L. (1986) Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology* **25**(4), 591-600

Pauciullo, A., Nicodemo, D., Castello, A., Cosenza, G., Ramunno, L., Rubes, J., and Di Berardino, D. (2009) Chromosomal unbalancements in sperm and oocytes of two Italian cattle breeds as determined by dual color fluorescent in situ hybridization (FISH). *Italian Journal of Animal Science* **8**(SUPPL. 2), 123-125

Pauciullo, A., Nicodemo, D., Cosenza, G., Peretti, V., Iannuzzi, A., Di Meo, G.P., Ramunno, L., Iannuzzi, L., Rubes, J., and Di Berardino, D. (2012a) Similar rates of chromosomal aberrant secondary oocytes in two indigenous cattle (Bos taurus) breeds as determined by dual-color FISH. *Theriogenology* **77**(3), 675-683

Pauciullo, A., Nicodemo, D., Peretti, V., Marino, G., Iannuzzi, A., Cosenza, G., Di Meo, G.P., Ramunno, L., Iannuzzi, L., Rubes, J., and Di Berardino, D. (2012b) X-Y aneuploidy rate in sperm of two "minor" breeds of cattle (Bos taurus) by using dual color fluorescent in situ hybridization (FISH). *Theriogenology* **78**(3), 688-695

Pavlok, A. (2000) D-penicillamine and granulosa cells can effectively extend the fertile life span of bovine frozen-thawed spermatozoa in vitro: Effect on fertilization and polyspermy. *Theriogenology* **53**(5), 1135-1146

Pavlok, A., Kubelka, M., and Pěknicová, J. (2001) The effect of various capacitation active compounds and capacitation time on the in vitro fertility and protein tyrosine phosphorylation profiles of bovine sperm. *Zygote* **9**(1), 25-38

Peñagaricano, F., and Khatib, H. (2012) Association of milk protein genes with fertilization rate and early embryonic development in Holstein dairy cattle. *J Dairy Res* **79**(1), 47-52

Pereira, M.M., MacHado, M.A., Costa, F.Q., Serapiao, R.V., Viana, J.H.M., and Camargo, L.S.A. (2010) Effect of oxygen tension and serum during IVM on developmental competence of bovine oocytes. *Reproduction, Fertility and Development* **22**(7), 1074-1082

Phocas, F., Boivin, X., Sapa, J., Trillat, G., Boissy, A., and Le Neindre, P. (2006) Genetic correlations between temperament and breeding traits in Limousin heifers. *Anim Sci* **82**(6), 805-811

Pontes, J.H.F., Nonato-Junior, I., Sanches, B.V., Ereno-Junior, J.C., Uvo, S., Barreiros, T.R.R., Oliveira, J.A., Hasler, J.F., and Seneda, M.M. (2009) Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. *Theriogenology* **71**(4), 690-697

Pontes, J.H.F., Silva, K.C.F., Basso, A.C., Rigo, A.G., Ferreira, C.R., Santos, G.M.G., Sanches, B.V., Porcionato, J.P.F., Vieira, P.H.S., Faifer, F.S., Sterza, F.A.M., Schenk, J.L., and Seneda, M.M. (2010) Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. *Theriogenology* **74**(8), 1349-1355

Ptak, G., Tischner, M., Bernabo, N., and Loi, P. (2003) Donor-dependent developmental competence of oocytes from lambs subjected to repeated hormonal stimulation. *Biol Reprod* **69**(1), 278-285

Purpera, M.N., Giraldo, A.M., Ballard, C.B., Hylan, D., Godke, R.A., and Bondioli, K.R. (2009) Effects of culture medium and protein supplementation on mRNA expression of in vitro produced bovine embryos. *Molecular Reproduction and Development* **76**(8), 783-793

Rambags, B.P.B., Krijtenburg, P.J., Van Drie, H.F., Lazzari, G., Galli, C., Pearson, P.L., Colenbrander, B., and Stout, T.A.E. (2005) Numerical chromosomal abnormalities in equine embryos produced in vivo and in vitro. *Molecular Reproduction and Development* **72**(1), 77-87

Rasmussen, S., Block, J., Seidel, G.E., Brink, Z., McSweeney, K., Farin, P.W., Bonilla, L., and Hansen, P.J. (2013) Pregnancy rates of lactating cows after transfer of in vitro produced embryos using X-sorted sperm. *Theriogenology* **79**(3), 453-461

Rátky, J., Brüssow, K.P., Egerszegi, I., Torner, H., Schneider, F., Solti, L., and Manabe, N. (2005) Comparison of follicular and oocyte development and reproductive hormone secretion during the ovulatory period in Hungarian native breed, Mangalica, and Landrace gilts. *J Reprod Develop* **51**(4), 427-432

Räty, M., Ketoja, E., Pitkänen, T., Ahola, V., Kananen, K., and Peippo, J. (2011) In vitro maturation supplements affect developmental competence of bovine cumulus-oocyte complexes and embryo quality after vitrification. *Cryobiology* **63**(3), 245-255

Ribeiro, L.V.P., Rigolon, L.P., Cavalieri, F.L.B., Seko, M.B., Martinez, A.C., Ribeiro, M.G., Martins, R.R., Ávila, M.R., and de Conti, J.B. (2011) Oocyte recovery and in vitro production from cows stimulated with either FSH or eCG. *Arch Zoot* **60**(232), 1021-1029

Rizos, D., Ward, F., Duffy, P., Boland, M.P., and Lonergan, P. (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development* **61**(2), 234-248

Rodero, E., González, A., Luque, M., Herrera, M., and Gutiérrez-Estrada, J.C. (2011) Classification of Spanish autochthonous bovine breeds. Morphometric study using classical and heuristic techniques. *Livestock Science*

Rodero Serrano, E., and Valera Córdoba, M. (Eds) (2008) 'La Ganadería Andaluza en el siglo XXI.' Patrimonio Ganadero Andaluz (Junta de Andalucía: Sevilla)

Rodriguez-Martinez, H. (2012) Assisted reproductive techniques for cattle breeding in developing countries: A critical appraisal of their value and limitations. *Reproduction in Domestic Animals* **47**(SUPPL. 1), 21-26

Rodriguez-Martinez, H., and Barth, A.D. (2007) In vitro evaluation of sperm quality related to in vivo function and fertility. *Society of Reproduction and Fertility supplement* **64**, 39-54

Rodriguez, P.C., Satorre, M.M., and Beconi, M.T. (2012) Effect of two intracellular calcium modulators on sperm motility and heparin-induced capacitation in cryopreserved bovine spermatozoa. *Anim Reprod Sci* **131**(3-4), 135-142

Rose-Hellekant, T.A., Libersky-Williamson, E.A., and Bavister, B.D. (1998) Energy substrates and amino acids provided during in vitro maturation of bovine oocytes alter acquisition of developmental competence. *Zygote* **6**(4), 285-294

Rose, T.A., and Bavister, B.D. (1992) Effect of oocyte maturation medium on in vitro development of in vitro fertilized bovine embryos. *Mol Reprod Develop* **31**(1), 72-77

Russell, D.F., Baqir, S., Bordignon, J., and Betts, D.H. (2006a) The impact of oocyte maturation media on early bovine embryonic development. *Molecular Reproduction and Development* **73**(10), 1255-1270

Russell, D.F., Baqir, S., Bordignon, J., and Betts, D.H. (2006b) The impact of oocyte maturation media on early bovine embryonic development. *Mol Reprod Dev* **73**(10), 1255-1270

Rybar, R., Kopecka, V., Prinosilova, P., Kubickova, S., Veznik, Z., and Rubes, J. (2010) Fertile bull sperm aneuploidy and chromatin integrity in relationship to fertility. *International Journal of Andrology* **33**(4), 613-622

Sakaguchi, M., Dominko, T., Leibfried-Rutledge, M.L., Nagai, T., and First, N.L. (2000) A combination of EGF and IGF-I accelerates the progression of meiosis in bovine follicular oocytes in vitro and fetal calf serum neutralizes the acceleration effect. *Theriogenology* **54**(8), 1327-1342

Santos, F.A., Neves, A.C., Guerra, M.P., and Reis, J.D.C. (2008) Superovolatory response in Saint Ines breed in Amazonian region of Brazil. *Reproduction in Domestic Animals* **43**, 86-86

Sartori, R., Bastos, M.R., and Wiltbank, M.C. (2010) Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. *Reprod Fert Develop* **22**(1), 151-158

Schmidt-Hebbel, J., Toniollo, G.H., Leite, F.G., Ferraudo, A.S., Perecin, D., and Pacola, L.J. (2000) Physical and morfhological semen characteristics in Gir, Guzera and Nelore (Bos taurus indicus) and Caracu (Bos taurus taurus) young bulls. *Características físicas e morfológicas de sêmen de touros jovens das raças Gir, Guzerá, Nelore (Bos taurus indicus) e Caracu (Bos taurus taurus)* **52**(5), 461-467

Shahani, S.K., Revell, S.G., Argo, C.G., and Murray, R.D. (2010) Mid-piece length of spermatozoa in different cattle breeds and its relationship to fertility. *Pakistan Journal of Biological Sciences* **13**(16), 802-808

Shamsuddin, M., Rodriguez-Martinez, H., and Larsson, B. (1993) Fertilizing capacity of bovine spermatozoa selected after swim-up in hyaluronic acid-containing medium. *Reprod Fertil Dev* **5**(3), 307-15

Sharma, R.K., Seifarth, K., Garlak, D., and Agarwal, A. (1999) Comparison of three sperm preparation media. *International Journal of Fertility and Women's Medicine* **44**(3), 163-167

Silva, B., Gonzalo, A., and Cañón, J. (2006) Genetic parameters of aggressiveness, ferocity and mobility in the fighting bull breed. *Anim Res* **55**(1), 65-70

Singh, S., Gupta, H.P., Misra, A.K., and Saxena, M.S. (2009) Determination of suitable concentration of caffeine and incubation period for in vitro capacitation of bovine spermatozoa. *Indian Journal of Animal Research* **43**(1), 17-21

Sirard, M.A., Parrish, J.J., Ware, C.B., Leibfried-Rutledge, M.L., and First, N.L. (1988) The culture of bovine oocytes to obtain developmentally competent embryos. *Biology of Reproduction* **39**(3), 546-552

Smetanina, I.G., Tatarinova, L.V., and Krivokharchenko, A.S. (2006) In vitro fertilization of bovine oocytes in protein-free culture system. *Russian Journal of Developmental Biology* **37**(6), 366-370

Söderquist, L., Janson, L., Håård, M., and Einarsson, S. (1996) Influence of season, age, breed and some other factors on the variation in sperm morphological abnormalities in Swedish dairy A.I. bulls. *Anim Reprod Sci* **44**(2), 91-98

Solti, L., Crichton, E.G., Loskutoff, N.M., and Cseh, S. (2000) Economical and ecological importance of indigenous livestock and the application of assisted reroduction to their preservation. *Theriogenology* **53**(1), 149-162

Somfai, T., Bodó, S., Nagy, S., Papp, Á.B., Iváncsics, J., Baranyai, B., Gócza, E., and Kovács, A. (2002) Effect of swim up and Percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. *Reproduction in Domestic Animals* **37**(5), 285-290

Squires, E.L. (2005) Integration of future biotechnologies into the equine industry. *Anim Reprod Sci* **89**(1-4 SPEC. ISS.), 187-198

Sugimura, S., Akai, T., Hashiyada, Y., Somfai, T., Inaba, Y., Hirayama, M., Yamanouchi, T., Matsuda, H., Kobayashi, S., Aikawa, Y., Ohtake, M., Kobayashi, E., Konishi, K., and Imai, K. (2012) Promising system for selecting healthy in vitro-fertilized embryos in cattle. *PLoS ONE* **7**(5)

Sumantri, C., Boediono, A., Ooe, M., Murakami, M., Saha, S., and Suzuki, T. (1997) The effect of sperm-oocyte incubation time on in vitro embryo development using sperm from a tetraparental chimeric bull. *Anim Reprod Sci* **48**(2-4), 187-195

Suzuki, H., Saito, Y., Kagawa, N., and Yang, X. (2003) In vitro fertilization and polyspermy in the pig: Factors affecting fertilization rates and cytoskeletal reorganization of the oocyte. *Microscopy Research and Technique* **61**(4), 327-334

Tajik, P., Beheshti-Govij, R., Soleimani-Rad, J., Ghasemzadeh-Nava, H., and Mohamadi-Roshandeh, A. (2008) Effects of different concentrations of bovine follicular fluid and estrous cow serum on development of murine 2-cell embryos. *Pakistan Journal of Biological Sciences* **11**(4), 628-632

Tajik, P., and Esfandabadi, N.S. (2003) In vitro maturation of caprine oocytes in different culture media. *Small Ruminant Research* **47**(2), 155-158

Takahashi, K., Matsui, H., Takahashi, I., Matsumoto, H., Fukui, E., Motoyama, M., and Yoshizawa, M. (2010) Effects of in vitro aging of mouse oocytes on metaphase ii spindle morphology, in vitro fertilization and subsequent embryonic development. *Journal of Mammalian Ova Research* **27**(1), 42-50

Tanghe, S., Van Soom, A., Sterckx, V., Maes, D., and De Kruif, A. (2002) Assessment of different sperm quality parameters to predict in vitro fertility of bulls. *Reproduction in Domestic Animals* **37**(3), 127-132

Tarín, J.J. (1996) Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Molecular Human Reproduction* **2**(10), 717-724

Tarín, J.J., Trounson, A.O., and Sathananthan, H. (1999) Origin and ploidy of multipronuclear zygotes. *Reproduction, Fertility and Development* **11**(4-5), 273-279

Tarkowsky, A. (1966) An air drying method for chromosome preparation of mouse eggs. *Cytogenetics* **5**, 294-300

Teixeira, H.C.A., Nascimento, N.V., McManus, C., Egito, A.A., Mariante, A.D.S., and Ramos, A.F. (2011) Seasonal influence on semen traits and freezability from locally adapted Curraleiro bulls. *Anim Reprod Sci* **125**(1-4), 56-61

Telenius, H., Pelmear, A.H., Tunnacliffe, A., Carter, N.P., Behmel, A., Ferguson- Smith, M.A., Nordenskjold, M., Pfragner, R., and Ponder, B.A.J. (1992) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow- sorted chromosomes. *Genes Chromosomes and Cancer* **4**(3), 257-263

Thibier, M. (2005) The zootechnical applications of biotechnology in animal reproduction: Current methods and perspectives. *Reproduction Nutrition Development* **45**(3), 235-242

Thundathil, J., Gil, J., Januskauskas, A., Larsson, B., Soderquist, L., Mapletoft, R., and Rodriguez-Martinez, H. (1999) Relationship between the proportion of capacitated spermatozoa present in frozen-thawed bull semen and fertility with artificial insemination. *International Journal of Andrology* **22**(6), 366-373

Ueno, S., Kurome, M., Ueda, H., Tomii, R., Hiruma, K., and Nagashima, H. (2005) Effects of maturation conditions on spindle morphology in porcine MII oocytes. *Journal of Reproduction and Development* **51**(3), 405-410

Ulloa Ulloa, C.M., Yoshizawa, M., Komoriya, E., Mitsui, A., Nagai, T., and Kikuchi, K. (2008a) The blastocyst production rate and incidence of chromosomal abnormalities by developmental stage in in vitro produced porcine embryos. *Journal of Reproduction and Development* **54**(1), 22-29

Ulloa Ulloa, C.M., Yoshizawa, M., Yamashita, A., Hama, S., Mitsui, A., Hashi, C., Abe, H., Hoshi, H., Fukui, E., and Matsumoto, H. (2008b) Blastocyst production from in vitro-produced day-2 bovine embryos classified by cleavage stage, and cytogenetical evaluation of the resultant day-8 blastocysts. *Journal of Reproduction and Development* **54**(6), 465-472

Vadnais, M.L., Kirkwood, R.N., Specher, D.J., and Chou, K. (2005) Effects of extender, incubation temperature, and added seminal plasma on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. *Anim Reprod Sci* **90**(3-4), 347-54

Van Soom, A., and De Kruif, A. (1996) Oocyte maturation, sperm capacitation and pre-implantation development in the bovine: Implications for in vitro production of embryos. *Reproduction in Domestic Animals* **31**(6), 687-701

Van Soom, A., Van Vlaenderen, I., Mahmoudzadeh, A.R., Ysebaert, M.T., and De Kruif, A. (1993) Salvage of oocytes from sterile genetically valuable cows, resulting in the birth of a calf. *Anim Reprod Sci* **36**(3-4), 187-196

Van Soom, A., Vandaele, L., Goossens, K., de Kruif, A., and Peelman, L. (2007) Gamete origin in relation to early embryo development. *Theriogenology* **68 Suppl 1**, S131-7

Vandaele, L., and Van Soom, A. (2011) Intrinsic factors affecting apoptosis in bovine in vitro produced embryos. *Verhandelingen - Koninklijke Academie voor Geneeskunde van België* **73**(1-2), 79-104

Vegetti, W., Van Assche, E., Frias, A., Verheyen, G., Bianchi, M.M., Bonduelle, M., Liebaers, I., and Van Steirteghem, A. (2000) Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence in-situ hybridization in infertile men. *Human Reproduction* **15**(2), 351-365

Vialard, F., Boitrelle, F., Molina-Gomes, D., and Selva, J. (2011) Predisposition to aneuploidy in the oocyte. *Cytogenetic and Genome Research* **133**(2-4), 127-135

Viana, J.H.M., De Almeida Camargo, L.S., De Moraes Ferreira, A., De Sa, W.F., De Carvalho Fernandes, C.A., and De Pinho Marques Jr, A. (2004) Short intervals between ultrasonographically guided follicle aspiration improve oocyte quality but do not prevent establishment of dominant follicles in the Gir breed (Bos indicus) of cattle. *Animal Reproduction Science* **84**(1-2), 1-12

Viana, J.H.M., Palhao, M.P., Siqueira, L.G.B., Fonseca, J.F., and Camargo, L.S.A. (2010a) Ovarian follicular dynamics, follicle deviation, and oocyte yield in Gyr breed (Bos indicus) cows undergoing repeated ovum pick-up. *Theriogenology* **73**(7), 966-972

Viana, J.H.M., Siqueira, L.G.B., Palhão, M.P., and Camargo, L.S.D.A. (2010b) Use of in vitro fertilization technique in the last decade and its effect on Brazilian embryo industry and animal production. *Acta Scientiae Veterinariae* **38**(SUPPL. 2), s661-s674

Villagómez, D.A.F., Parma, P., Radi, O., Di Meo, G., Pinton, A., Iannuzzi, L., and King, W.A. (2009) Classical and molecular cytogenetics of disorders of sex development in domestic animals. *Cytogenetic and Genome Research* **126**(1-2), 110-131

Villamediana, P., Vidal, F., and Paramio, M.T. (2001) Cytogenetic analysis of caprine 2- to 4-cell embryos produced in vitro. *Zygote* **9**(3), 193-9

Visconti, P.E., and Kopf, G.S. (1998) Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod* **59**(1), 1-6

Viuff, D., Greve, T., Avery, B., Hyttel, P., Brockhoff, P.B., and Thomsen, P.D. (2000) Chromosome aberrations in in vitro-produced bovine embryos at days 2-5 post-insemination. *Biology of Reproduction* **63**(4), 1143-1148

Viuff, D., Hendriksen, P.J.M., Vos, P.L.A.M., Dieleman, S.J., Bibbiy, B.M., Greve, T., Hyttel, P., and Thomsen, P.D. (2001) Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. *Biology of Reproduction* **65**(1), 204-208

Viuff, D., Palsgaard, A., Rickords, L., Lawson, L.G., Greve, T., Schmidt, M., Avery, B., Hyttel, P., and Thomsen, P.D. (2002) Bovine embryos contain a higher proportion of polyploid cells in the trophectoderm than in the embryonic disc. *Molecular Reproduction and Development* **62**(4), 483-488

Viuff, D., Rickords, L., Offenberg, H., Hyttel, P., Avery, B., Greve, T., Olsaker, I., Williams, J.L., Callesen, H., and Thomsen, P.D. (1999) A high proportion of bovine blastocysts produced in vitro are mixoploid. *Biology of Reproduction* **60**(6), 1273-1278

Waberski, D., Petrounkina, A., Weitze, K.F., and Töpfer-Petersen, E. (1999) In vitro assessment of semen for the prediction of fertility. *In-vitro-Beurteilung von Sperma zur Vorhersage der Fertilität* **27**(1), 1-7

Walsh, S.W., Williams, E.J., and Evans, A.C.O. (2011) A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci* **123**(3-4), 127-138

Wall, R.J., Kerr, D.E., and Bondioli, K.R. (1997) Transgenic Dairy Cattle: Genetic Engineering on a Large Scale. *Journal of Dairy Science* **80**(9), 2213-2224

Wang, W.H., Hosoe, M., and Shioya, Y. (1997) Induction of cortical granule exocytosis of pig oocytes by spermatozoa during meiotic maturation. *Journal of Reproduction and Fertility* **109**(2), 247-255

Wang, Z.G., Yu, S.D., and Xu, Z.R. (2007) Effects of collection methods on recovery efficiency, maturation rate and subsequent embryonic developmental competence of oocytes in holstein cow. *Asian Austral J Anim* **20**(4), 496-500

Ward, C.R., and Storey, B.T. (1984) Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. *Developmental Biology* **104**(2), 287-296

Ward, F., Enright, B., Rizos, D., Boland, M., and Lonergan, P. (2002) Optimization of in vitro bovine embryo production: Effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology* **57**(8), 2105-2117

Ward, F., Rizos, D., Corridan, D., Quinn, K., Boland, M., and Lonergan, P. (2001) Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo. *Molecular Reproduction and Development* **60**(1), 47-55

Way, A.L., and Killian, G.J. (2002) Capacitation and induction of the acrosome reaction in bull spermatozoa with norepinephrine. *Journal of Andrology* **23**(3), 352-357

Wells, D., and Levy, B. (2003) Cytogenetics in reproductive medicine: The contribution of comparative genomic hybridization (CGH). *BioEssays* **25**(3), 289-300

Wheeler, M.B., and Seidel Jr, G.E. (1989) Capacitation of bovine spermatozoa by lysophospholipids and trypsin. *Gamete Research* **22**(2), 193-204

Whitfield, C.H., and Parkinson, T.J. (1992) Relationship between fertility of bovine semen and In vitro induction of acrosome reactions by heparin. *Theriogenology* **38**(1), 11-20

Wilson, T.D. (1991) Monosomy and trisomy in bovine embryos sired by bulls heterozygous for the 1 29 Robertsonian translocation chromosome. *Theriogenology* **36**(5), 789-793

Wortzman, G.B., and Evans, J.P. (2005) Membrane and cortical abnormalities in post-ovulatory aged eggs: Analysis of fertilizability and establishment of the membrane block to polyspermy. *Molecular Human Reproduction* **11**(1), 1-9

Wu, B., and Zan, L. (2012) Enhance Beef Cattle Improvement by Embryo Biotechnologies. *Reproduction in Domestic Animals*

Wyrobek, A.J., Eskenazi, B., Young, S., Arnheim, N., Tiemann-Boege, I., Jabs, E.W., Glaser, R.L., Pearson, F.S., and Evenson, D. (2006) Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proceedings of the National Academy of Sciences of the United States of America* **103**(25), 9601-9606

Xu, L., Wang, Y., Zhou, P., Cao, Y.X., Huang, T.H., and Chian, R.C. (2008) Cytogenetic analysis of in vivo and in vitro matured oocytes derived from naturally cycling and stimulated mice. *Systems Biology in Reproductive Medicine* **54**(3), 155-162

Yang, X., Jiang, S., and Foote, R.H. (1993) Bovine oocyte development following different oocyte maturation and sperm capacitation procedures. *Molecular Reproduction and Development* **34**(1), 94-100

Young, M.W., Tiedemann, G., Pepin, R.A., Turner, J.D., Okita, W.B., Harvey, M., Groet, S., and Curling, J.M. (1998) Transgenics go commercial. *BioPharm* **11**(7), 24-28

Younis, A.I., Brackett, B.G., and Fayrer-Hosken, R.A. (1989) Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. *Gamete Res* **23**(2), 189-201

Zenzes, M.T., and Casper, R.F. (1992) Cytogenetics of human oocytes, zygotes, and embryos after in vitro fertilization. *Human Genetics* **88**(4), 367-375

Zheng, P., and Dean, J. (2007) Oocyte-Specific Genes Affect Folliculogenesis, Fertilization, and Early Development. *Semin Reprod Med* **25**(4), 243-251

Zuelke, K.A. (1998) Transgenic modification of cows milk for value-added processing. *Reproduction, Fertility and Development* **10**(8), 671-676

Anexo – Artículos Publicados

Annex – Published articles

Capítulo Dos / Chapter Two

Livestock Science 149 (2012) 275–281

Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

In *vitro* induction of the acrosome reaction in spermatozoa from endangered Spanish bulls: Effect of breed, culture media and incubation time

S. Demyda-Peyras [a,c,*], J. Dorado [b], M. Hidalgo [b], L. De Luca [a], A. Muñoz-Serrano [c], M. Moreno-Millan [c]

[a] Faculty of Agricultural Sciences, Lomas de Zamora University, 1836 Buenos Aires, Argentina
[b] Animal Reproduction Group, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, 14071 Cordoba, Spain
[c] Department of Genetics, Faculty of Veterinary Medicine, University of Cordoba, 14071 Cordoba, Spain

ARTICLE INFO

Article history:
Received 25 February 2012
Received in revised form
26 June 2012
Accepted 23 July 2012

Keywords:
Cattle breed
Acrosome reaction
Cryopreserved sperm
Fluorescent techniques

ABSTRACT

Current conservation programs for endangered species have been widely benefited from the use of modern assisted reproductive techniques. However, at the present time, there is little information available regarding the use of locally adapted cattle breeds on IVF programs, and even less about their sperm behavior. The aims of this study were to (1) evaluate standard acrosome reaction (AR) protocols in cryopreserved semen doses of Andalusian endangered cattle breeds, and (2) investigate the effect of breed, culture medium supplementation and incubation time on AR test results. To this end, 80 frozen semen doses from 16 bulls (5 semen samples per bull) of five different cattle breeds (3 Berrendo en Colorado, 6 Berrendo en Negro, 2 Cardena Andaluza, 1 Pajuna and 4 Retinta) were in vitro incubated in three test media (Heparin, BSA or their combination), using Sp-TALP as a control, to induce in vitro AR. The percentage of acrosome-reacted spermatozoa was evaluated using fluorescence techniques (FITC-PNA and Propidium Iodide) at four time-points (0, 30, 60 or 120 min.). Throughout the whole experiment, spermatozoa incubated with a combination of both supplements (Heparin and BSA) showed significantly ($P<0.05$) higher results than those processed with Heparin or BSA alone. In contrast, the control media (without supplementation) showed the lowest results. We also found a significant ($P<0.05$) influence of cattle breed on the dynamics of the in vitro induced AR using different protocols. Based on our results, we can conclude that the response pattern of in vitro induced AR depends not only on culture media and incubation time but also on the cattle breed. Therefore, all these factors must be taken in consideration to assess the rate of AR sperm in bulls.

1. Introduction

Extensive cattle production in the Spanish region of Andalusia is based on autochthonous breeds well adapted to grazing conditions (Rodero Serrano and Valera Córdoba, 2008). There is ample evidence that livestock breeds and populations evolved over the years in stressful environments have a range of unique adaptive traits (e.g. disease resistance, physiological and behavioral adaptations) which enable them to be productive in these environments (Mirkena et al., 2010). Limitations on these breeds usually are both the low animal number available for reproductive purposes and the high level of inbreeding existing in their herds. Current conservation programs for endangered

* Corresponding author at: Department of Genetics, Faculty of Veterinary Medicine, University of Cordoba, Gregor Mendel Building, Campus de Rabanales, Carretera Madrid-Cádiz, Km 396, 14071 Cordoba, Spain. Tel.: +34 957 21 85 09; fax: +34 957 21 87 07.
E-mail address: ge2depes@uco.es (S. Demyda-Peyras).

1871-1413/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.livsci.2012.07.025

species have been widely benefited from the use of modern assisted reproductive techniques, such as the *in vitro* embryo technology (IVF) (Andrabi and Maxwell, 2007; Solti et al., 2000). However, at the present time, there is little information available regarding the use of locally adapted cattle breeds on IVF programs and even less about their sperm behavior (Teixeira et al., 2011).

Recently, Hoflack et al. (2006) has recommended a routine semen evaluation consisting of semen volume, sperm concentration, motility and morphology prior to include semen donor bulls into an assisted reproduction program. However, it has been demonstrated that bulls with good sperm motility parameters can have a reduced fertilization capacity due to a failure of the acrosome reaction of sperm (Lessard et al., 2011). It may suggest that the classical semen parameters might not be enough to assess the fertilizing capacity of a semen sample. Previous studies have suggested the use of *in vitro* acrosome reaction (AR) test as a valuable tool to predict both field fertility (Birck et al., 2010; Waberski et al., 1999) and *in* vitro fertilizing capacity of semen in cattle (Costa et al., 2010; Molnarova et al., 2006).

Those procedures to induce AR are date back a long time (Parrish et al., 1988). Likewise, heparin and bovine serum albumin (BSA) have been widely used as AR inducers, mainly due to their neutral effect on the oocyte and sperm vitality (Parrish et al., 1999). Interacting with these compounds, the incubation time also has an important role in AR tests (Pavlok et al., 2001). In fact, it has been widely demonstrated that the percentage of acrosome-reacted spermatozoa increases during the incubation time (Hochi et al., 1996; Parrish et al., 1988). However, to our knowledge, there are no studies on the acrosome reaction-inducing activity of either heparin, BSA or their combination in Spanish endangered cattle breeds.

The aims of this study were therefore to (1) evaluate standard AR protocols in cryopreserved semen doses of Andalusian endangered cattle breeds, as a condition for its potential use in IVF programs, and (2) investigate the effect of breed, culture medium supplementation and incubation time on AR test results.

2. Materials and methods

2.1. Semen samples

In total, 80 frozen semen samples from 16 bulls (5 semen samples per bull) of five Andalusian endangered native breeds: 3 Berrendo en Colorado (BC), 6 Berrendo en Negro (BC), 2 Cardena Andaluza (CA), 1 Pajuna (PA) and 4 Retinta (RE) were analyzed in this study. Semen collection and freezing were performed at the Animal Reproduction, Selection and Conservation Center—CENSYRA, (Badajoz, Spain), as described by Januskauskas et al. (1996). Only samples with acceptable post-thawing viability and motility ($>40\%$ viable sperm and $>50\%$ progressively motile sperm) were used in this experiment (Birck et al., 2010).

2.2. Sperm selection

Frozen semen doses were thawed in a water bath at 37 °C for 1 min. Viable spermatozoa were selected by "Swim Up" method as described previously by Shamsuddin et al. (1993) Briefly, aliquots of 500 µl of semen were carefully placed at the bottom of a 2 ml tube containing 1 ml of equilibrated Sp-TALP medium (Sp-T, Caisson Laboratories, USA) and incubated for 1 h at 39 °C, in a 5% CO_2 atmosphere. After incubation, 1 ml upper fraction was collected and mixed with 5 ml of equilibrated Sp-T in a conical tube and centrifuged (150g, 10 min. at 24 °C). The supernatant was discarded and the sperm pellet was resuspended in 3 ml of Sp-T and centrifuged again (150g, 10 min at 24 °C). Thereafter, the sperm pellet was transferred to a clean conical tube containing Sp-T medium.

2.3. Acrosome reaction status

Acrosome integrity was assessed as described by Cheng et al. (1996), with minor modifications. In brief, aliquots (50 µl) of semen samples were diluted in PBS to a final concentration of 25×10^6 spermatozoa/ml. Droplets (10 µl) of the sperm sample smeared onto a microscopic slide were air dried, fixed, and permeabilised with 70% (v:v) ethanol for 30 s. A mixture of 10 µl of propidium iodide (0.1 mg/mL) and 20 µl of isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (0.1 mg/mL) was then spread over each smear. Slides were incubated in a dark and moist chamber at 4 °C for 30 min. They were subsequently rinsed with excess deionized water at 4 °C mounted with 10 µl of VECTASHIELD® Mounting Media (Vector Laboratories Ltd., Peterborough, United Kingdom), covered with a coverslip, and sealed with nail polish. A total of 100 spermatozoa were counted per slide under $400\times$ magnification with an epifluorescence microscopy (Reichert-Jung Polyvar, Austria) using a BP 460–490 nm excitation filter. Two sperm subpopulations were identified: (1) spermatozoa displaying intensively green fluorescence of the acrosomal cap indicated an intact outer acrosomal membrane, and (2) spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap indicated the process of vesiculation and breakdown of the acrosomal membrane, or spermatozoa displaying a fluorescent band at the equatorial segment or no fluorescence indicated residues or a complete loss of the outer acrosomal membrane, respectively. Values were expressed as percentages.

2.4. Experimental design

Eighty frozen semen samples from 16 bulls (5 semen samples per bull) of five different endangered breeds (3 BC, 6 BN, 2 CA, 1 PA and 4 RE) were used in this study. Swim up selected spermatozoa were incubated at 38 °C in a 5% CO_2 atmosphere, in four different culture media: Sp-TALP medium (Sp-T; Caisson Laboratories, USA), Sp-TALP medium supplemented with 0.6% BSA (Sigma Aldrich Spain, A3311) (Sp-TB), Sp-TALP medium supplemented with 10 µg/ml of Heparin (Sigma Aldrich, Spain, H3149) (Sp-TH), and Sp-TALP medium supplemented with 0.6% BSA and 10 µg/ml of Heparin (Sp-TBH). Finally, aliquots were collected at 0, 30, 60 and 120 min. and stained for AR evaluation. Five replications were made.

2.5. Statistical analysis

A three-fixed factor design was performed: *animal breed* (BC, BN, CA, PA and RE), *culture media* (Sp-T, Sp-TB, Sp-TH

and Sp-TBH) and *incubation time* (T0, T30, T60 and T120). Data were tested for normal distribution using the Kolmogorov–Smirnov test prior to ANOVA, and analyzed using the General Lineal Model (GLM) procedure of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). Individual factors and breed-culture medium, breed-time and time-culture medium interactions were also evaluated, taking into account the imbalance between the number of bulls among breeds. Duncan's Multiple Range procedure was performed to test differences among means. Data were significant at $P < 0.05$.

For each culture media and breed combination, the orthogonal contrast test was performed in order to analyze the interaction between the main effect and the interaction effects of the AR status over time.

3. Results

Significant ($P < 0.01$) effects of breed, culture medium and incubation time on AR-status were found. Moreover, significant ($P < 0.01$) interactions among all these factors were found. For this reason, each independent variable (breed, culture medium or incubation time) was then analyzed based on the other two.

3.1. Effect of the breed on the in vitro induced AR

As shown in Table 1, *in vitro*-induced AR in bull sperm in response to different culture media and incubation time varied significantly ($P < 0.01$) depending on the breed. The *in vitro* AR-induced by Sp-TH was consistent

Table 1
Effect of the cattle breed on the in vitro induced AR test in bull spermatozoa.

IT	Breed	Culture media			
		Sp-T	Sp-TB	Sp-TH	Sp-TBH
T30	BC	7.07 ± 0.34^{B}	7.53 ± 0.35^{A}	11.33 ± 0.44^{BC}	14.33 ± 0.30^{B}
	BN	5.13 ± 0.20^{A}	9.00 ± 0.29^{B}	12.40 ± 0.28^{C}	13.97 ± 0.24^{B}
	CA	4.80 ± 0.29^{A}	6.40 ± 0.37^{A}	8.10 ± 0.28^{A}	12.40 ± 0.37^{A}
	PA	5.80 ± 0.73^{A}	7.60 ± 0.68^{A}	10.40 ± 0.51^{B}	15.40 ± 0.51^{B}
	RE	7.53 ± 0.41^{B}	9.73 ± 0.43^{B}	11.60 ± 0.35^{BC}	17.80 ± 0.74^{C}
T60	BC	8.20 ± 0.20^{C}	13.80 ± 0.38^{AB}	14.00 ± 0.45^{A}	19.73 ± 0.45^{A}
	BN	6.67 ± 0.19^{AB}	14.50 ± 0.32^{AB}	14.63 ± 0.29^{AB}	22.93 ± 0.34^{B}
	CA	5.90 ± 0.28^{A}	13.20 ± 0.68^{A}	13.80 ± 0.59^{A}	22.00 ± 0.56^{B}
	PA	8.40 ± 0.40^{C}	19.20 ± 0.37^{C}	17.00 ± 0.45^{C}	28.00 ± 0.84^{C}
	RE	7.07 ± 0.33^{B}	14.73 ± 0.28^{B}	15.93 ± 0.37^{BC}	23.13 ± 0.48^{B}
T120	BC	9.07 ± 0.28^{A}	18.00 ± 0.44^{A}	20.73 ± 0.37^{A}	27.47 ± 0.38^{A}
	BN	11.30 ± 0.29^{B}	20.33 ± 0.22^{B}	23.30 ± 0.32^{B}	32.73 ± 0.39^{B}
	CA	8.70 ± 0.30^{A}	20.60 ± 0.45^{B}	22.80 ± 0.42^{B}	28.10 ± 0.35^{A}
	PA	10.40 ± 0.51^{B}	22.20 ± 0.37^{C}	20.40 ± 0.68^{A}	38.20 ± 1.02^{D}
	RE	9.20 ± 0.34^{A}	20.40 ± 0.40^{B}	21.87 ± 0.43^{AB}	36.27 ± 0.64^{C}

Culture media (CM): Sp-T:Sp-TALP; Sp-TB:Sp-TALP+BSA; Sp-TH:Sp-TALP+Heparin and Sp-TBH:Sp-TALP+BSA+Heparin. Incubation time (IT): T30: 30 min; T60: 60 min and T120: 120 min. Breed: BC: Berrendo en Colorado; BN: Berrendo en Negro; CA: Cardena Andaluza; PA: Pajuna and RE: Retinta. Values indicate the percentage of live acrosome-reacted spermatozoa expressed as mean ± S.E. On each block, values with different letters (A, B, C and D) are significantly different ($P < 0.05$, Duncan's test).

Table 2
Effect of the culture media on the in vitro induced AR in bull spermatozoa.

IT	CM	Cattle breed				
		BC	BN	CA	PA	RE
T30	Sp-T	7.07 ± 0.34^{A}	5.13 ± 0.20^{A}	4.80 ± 0.29^{A}	5.80 ± 0.73^{A}	7.53 ± 0.41^{A}
	Sp-TB	7.53 ± 0.35^{A}	9.00 ± 0.29^{B}	6.40 ± 0.37^{B}	7.60 ± 0.68^{A}	9.73 ± 0.43^{B}
	Sp-TH	11.33 ± 0.44^{B}	12.40 ± 0.28^{C}	8.10 ± 0.28^{C}	10.40 ± 0.51^{B}	11.60 ± 0.35^{C}
	Sp-TBH	14.33 ± 0.30^{C}	13.97 ± 0.24^{D}	12.40 ± 0.37^{D}	15.40 ± 0.51^{C}	17.80 ± 0.74^{D}
T60	Sp-T	8.20 ± 0.20^{A}	6.67 ± 0.19^{A}	5.90 ± 0.28^{A}	8.40 ± 0.40^{A}	7.07 ± 0.33^{A}
	Sp-TB	13.80 ± 0.38^{B}	14.50 ± 0.32^{B}	13.20 ± 0.68^{B}	19.20 ± 0.37^{C}	14.73 ± 0.28^{B}
	Sp-TH	14.00 ± 0.45^{B}	14.63 ± 0.29^{B}	13.80 ± 0.59^{B}	17.00 ± 0.45^{B}	15.93 ± 0.37^{C}
	Sp-TBH	19.73 ± 0.45^{C}	22.93 ± 0.34^{C}	22.00 ± 0.56^{C}	28.00 ± 0.84^{D}	23.13 ± 0.48^{D}
T120	Sp-T	9.07 ± 0.28^{A}	11.30 ± 0.29^{A}	8.70 ± 0.30^{A}	10.40 ± 0.51^{A}	9.20 ± 0.34^{A}
	Sp-TB	18.00 ± 0.44^{B}	20.33 ± 0.22^{B}	20.60 ± 0.45^{B}	20.40 ± 0.37^{B}	20.40 ± 0.40^{B}
	Sp-TH	20.73 ± 0.37^{C}	23.30 ± 0.32^{C}	22.80 ± 0.42^{C}	22.20 ± 0.68^{B}	21.87 ± 0.64^{C}
	Sp-TBH	27.47 ± 0.38^{D}	32.73 ± 0.39^{D}	28.10 ± 0.35^{D}	38.20 ± 1.02^{C}	36.27 ± 0.43^{D}

Culture media (CM): Sp-T:Sp-TALP; Sp-TB:Sp-TALP+BSA; Sp-TH:Sp-TALP+Heparin and Sp-TBH:Sp-TALP+BSA+Heparin. Incubation time (IT): T30: 30 min; T60: 60 min and T120: 120 min. Breed: BC: Berrendo en Colorado; BN: Berrendo en Negro; CA: Cardena Andaluza; PA: Pajuna and RE: Retinta. Values indicate the percentage of live acrosome-reacted spermatozoa expressed as mean ± S.E. On each block, values with different letters (A, B, C and D) are significantly different ($P < 0.05$, Duncan's test).

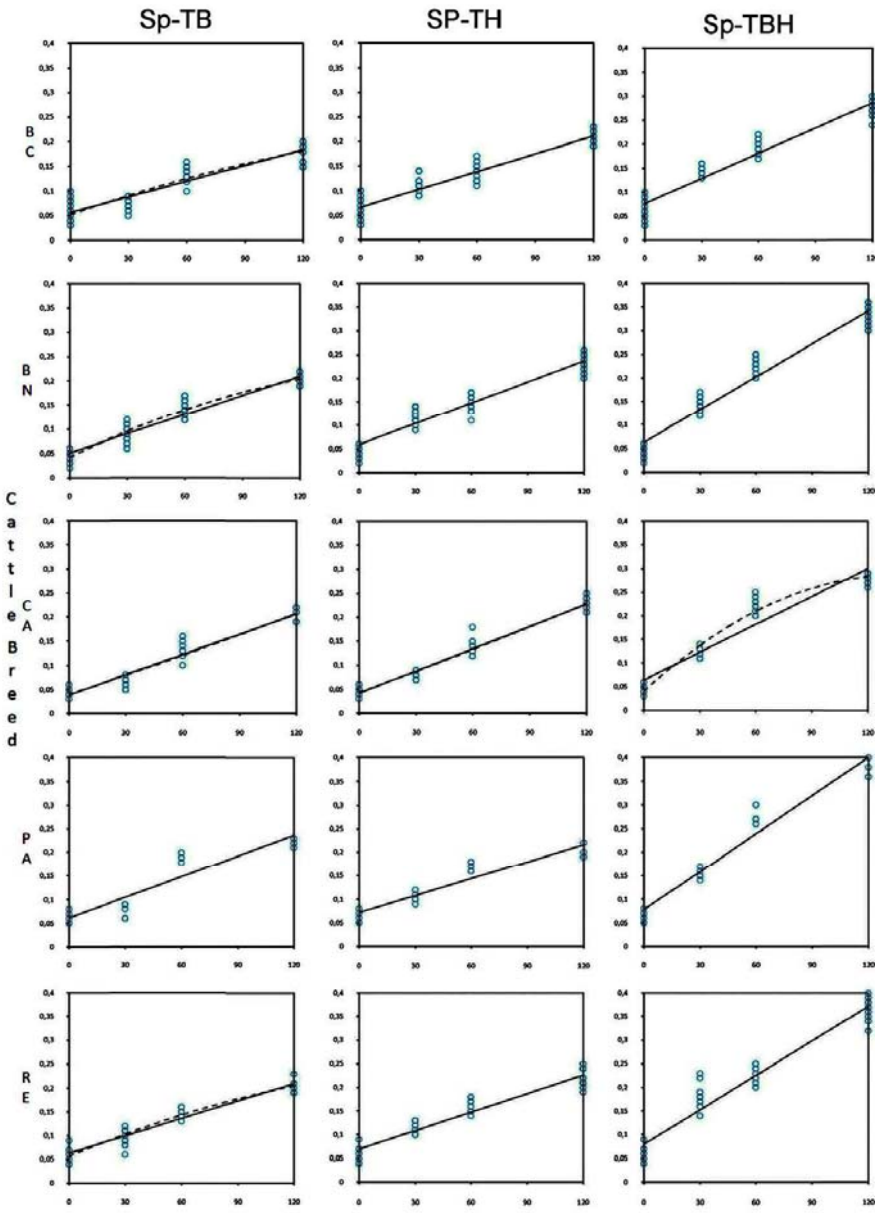


Fig. 1. Two-way (breed versus culture media) orthogonal contrast analysis of results obtained in the acrosome reaction test of frozen–thawe semen samples. The percentage of acrosome reacted spermatozoa fits to a linear function over time in all analyzed breeds (full line, $P < 0.01$). these breed media combinations (Sp-TB for BC, BN, CA and RE; Sp-TH for CA; and Sp-TBH for CA) were also fitted to a quadratic mode (do $P < 0.05$). Culture media: Sp-TB:Sp-TALP+BSA; Sp-TH:Sp-TALP+Heparin and Sp-TBH:Sp-TALP+BSA+Heparin. Breed: BC: Berrendo en Color Berrendo en Negro; CA: Cardena Andaluza; PA: Pajuna and RE: Retinta.

over time only in Retinta bulls (high mean AR values at T30, T60 and T120). For all the other breeds, a random pattern was observed for *in vitro*-induced AR.

3.2. Effect of the culture media on the in vitro induced AR

The percentage of induced AR for each culture medium is shown in Table 2. The AR status induced by each culture medium depends on both the breed and the incubation time, with exception of Sp-TBH. This culture medium showed the higher number of reacted sperm cells instead of breed and incubation time (Table 2). At T30 and T120, AR status was significantly ($P < 0.05$) higher in samples processed with Sp-TH compared with control medium (Sp-T) and Sp-TB, except for Pajuna bulls at T120. However, at T60, there was no significant ($P > 0.05$) differences between Sp-TH and Sp-TB for BC, BN and CA.

3.3. Effect of the incubation time on the in vitro induced AR

A positive trend was observed between AR status and incubation time regardless of the breed or the culture medium (Fig. 1). A significant ($P < 0.01$) linear relationship between *in vitro* induced AR in all breeds and culture media was shown by the orthogonal contrast analysis. However, some of these breed-media combinations (Sp-TB for BC, BN, CA and RE; Sp-TH for CA; and Sp-TBH for CA) were also fitted to a quadratic model.

4. Discussion

Effective IVF still needs as a prerequisite a good functional status of spermatozoa which must retain their ability to complete the acrosome reaction in a timely manner. The inability of spermatozoa to undergo the AR affects the male fertilizing potential under both *in vivo* and *in vitro* conditions, and may play a decisive role in unexplained low fertility (Birck et al., 2010). It has been previously demonstrated in several species that the percentage of the *in vitro*-induced AR is significantly lower in subfertile animals compared with fertile animals (Herrera et al., 2002; Meyers et al., 1996; Whitfield and Parkinson, 1992). For this reason, the study of this trait becomes important for high genetic value animals.

In general, the incidence of AR obtained *in vitro* in the current study is in consonance with those obtained by previous authors (Birck et al., 2010; Costa et al., 2010; Januskauskas et al., 2000; Parrish et al., 1988; Somfai et al., 2002), indicating that doses of semen stored at Andalusian Genetic Bank responded satisfactorily to *in vitro* AR test. We also observed that the incidence of spontaneous AR at 0 h was on average 6%. This result means that membrane damage due to freezing procedure was low in all studied samples, and thus they could be used in IVP programs (Kuroda et al., 2007). According with Coy et al. (2002), spontaneous acrosome-reacted spermatozoa are considered to be unable to fertilize oocytes. Moreover, the proportion of viable, uncapacitated spermatozoa present in semen has been previously shown to positively correlate with fertility in frozen–thawed semen samples (Thundathil et al., 1999) and may explain the appearance of clinical subfertility in cattle (Kuroda et al., 2007).

It is worth noting that the heparin added to culture medium increased the percentage of acrosome reacted spermatozoa in all studied breeds, as well as a synergistic effect was observed when heparin and BSA were added to culture medium (Sp-TBH). As documented by several authors (Arangasamy and Singh, 2007; Dapino et al., 2006; Parrish et al., 1988; Rodriguez et al., 2012), heparin has been shown to play a key role on *in vitro* sperm capacitation. Their initial contact with sperm induces the acrosome reaction by reorganizing membrane lipid domains, displacing spermatozoa surface proteins (Miller et al., 1990), stimulating the efflux of cholesterol and phospholipids, and increasing intracellular levels of calcium, pH and cAMP (Breininger et al., 2010). Moreover, cholesterol efflux has been identified as a fundamental proccess in the sperm acrosome reaction, resulting in an increase of the plasma membrane inestability (Cross, 1998). On the other hand, BSA assists in that process accepting cholesterol released by spermatozoa when it undergoes into AR (Kharche et al., 2009; Visconti and Kopf, 1998). In this sense, it has been previously demonstrated that the penetration rate of bovine oocytes processed in a protein free media was significantly lower than that obtained with BSA-supplemented media (Smetanina et al., 2006).

An unexpected result was that *in vitro* AR induced by the same incubation protocol was significantly different depending on the breed, suggesting differences between breeds in the response to AR test within cattle species. Although it has been previously demonstrated the influence of several paternal factors, such as male (Katska and Smorag, 1996; Ward et al., 2001), age (Ward et al., 2002), breed (Söderquist et al., 1996), species (Pontes et al., 2010), and semen collection method (García-Álvarez et al., 2009) on IVF outcomes, this is the first attempt to assess the effect of cattle breed on induced AR results. Early reports suggest that the ability of spermatozoa to undergo AR *in vitro* may be a useful method for predicting the result of IVF in cattle (Costa et al., 2010; Giritharan et al., 2005; Mukhopadhyay et al., 2008). In fact, AR status has been previously used as predictor of both freezability (Molnarova et al., 2006) and *in vivo* fertility (Birck et al., 2010; Collin et al., 2000; Giritharan et al., 2005) in cattle. However, it has been widely demonstrated that *in vitro* AR rates depend on both the incubation time (Dode et al., 2002; Fukui et al., 1990; Gliedt et al., 1996; Pavlok et al., 2001) and the culture medium (Januskauskas et al., 2000; Sharma et al., 1999; Vadnais et al., 2005). Our results are consistent with these previous observations, showing a clear interaction between culture medium and incubation time. However, these factors exerted differential effects on the AR status of bull spermatozoa for each of the studied breeds. This can partly be explained by breed differences in plasma membrane composition of bull spermatozoa (Casali et al., 1985). Due to this, the calculation of cattle fertility index from the outcome of AR test as suggested by previous authors (Costa et al., 2010; Molnarova et al., 2006; Mukhopadhyay et al., 2008) could not be recommended due to the effect exerted by breed, incubation protocol, and their interactions on the AR

status of bull spermatozoa. Nevertheless, low AR rates could be a cause of unexplained infertility (Lessard et al., 2011).

In conclusion, our results suggest that doses of stored semen of Andalusian cattle breeds respond satisfactorily to *in vitro* AR test, so that they could be used in future IVF programs. The response and pattern of the *in vitro* AR-induced depend not only on culture medium and incubation time but also on the cattle breed. Therefore, all these factors must be taken in consideration to assess the rate of AR sperm in bulls. Further experiments are needed to validate AR test protocols in different cattle breeds for its use as an accurate predictor of *in vitro* fertility and as a valuable tool for calculating a fertility index (ranking) among bulls of different breeds.

Conflict of interest statement

This letter is to declare that there was no conflict of interest in the study "*In vitro* induction of the acrosome reaction in spermatozoa from endangered Spanish bulls: Effect of breed, culture media and incubation time", presented for its evaluation in the Livestock Production Journal by any of the manuscript authors or their institution.

Acknowledgments

Semen samples were gently donated by Berrendo (ANABE), Cardena Andaluza, Pajuna and Retinta breeders associations. Sebastian Demyda is supported by a Grant from MAEC-AECID (Spain) and by University of Lomas de Zamora, Argentina (Project 13A178).

References

Andrabi, S.M.H., Maxwell, W.M.C., 2007. A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim. Reprod. Sci. 99, 223–243.
Arangasamy, A., Singh, L.P., 2007. In-vitro capacitation of epididymal spermatozoa with heparin and gelatin binding buffalo seminal plasma proteins. Indian J. Anim. Sci. 77, 549–552.
Birck, A., Christensen, P., Labouriau, R., Pedersen, J., Borchersen, S., 2010. In vitro induction of the acrosome reaction in bull sperm and the relationship to field fertility using low-dose inseminations. Theriogenology 73, 1180–1191.
Breininger, E., Cetica, P.D., Beconi, M.T., 2010. Capacitation inducers act through diverse intracellular mechanisms in cryopreserved bovine sperm. Theriogenology 74, 1036–1049.
Casali, E., Farruggia, G., Spisni, A., 1985. Isolation and enzymatic characterization of the plasmalemma from bovine spermatozoa. J. Exp. Zool. 235, 397–401.
Collin, S., Sirard, M.A., Dufour, M., Bailey, J.L., 2000. Sperm calcium levels and chlortetracycline fluorescence patterns are related to the in vivo fertility of cryopreserved bovine semen. J. Androl. 21, 938–943.
Costa, M.Z., Oliveira, L.Z., Resende, M.V., Lucio, A.C., Perini, A.P., Miguel, M.C.V., Lima, V.F.M.H., 2010. Induction of the Acrosome Reaction Test to In Vitro Estimate Embryo Production in Nelore Cattle. vol. 62, pp. 771–777.
Coy, P., Gadea, J., Romar, R., Matás, C., García, E., 2002. Effect of in vitro fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and in vitro development in pigs. Reproduction 124, 279–288.
Cross, N.L., 1998. Role of cholesterol in sperm capacitation. Biol. Reprod. 59, 7–11.
Cheng, F.P., Fazeli, A., Voorhout, W.F., Marks, A., Bevers, M.M., Colenbrander, B., 1996. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome eaction in stallion spermatozoa. J. Androl. 17, 674–682.
Dapino, D.G., Marini, P.E., Cabada, M.O., 2006. Effect of heparin on in vitro capacitation of boar sperm. Biol. Res. 39, 631–639.
Dode, M.A.N., Rodovalho, N.C., Ueno, V.G., Fernandes, C.E., 2002. The effect of sperm preparation and co-incubation time on in vitro fertilization of bos indicus oocytes. Anim. Reprod. Sci. 69, 15–23.
Fukui, Y., Sonoyama, T., Mochizuki, H., Ono, H., 1990. Effects of heparin dosage and sperm capacitation time on in vitro fertilization and cleavage of bovine oocytes matured in vitro. Theriogenology 34, 579–591.
García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J.J., Ramón, M., Fernández-Santos, M.R., Esteso, M.C., Pérez-Guzmán, M.D., Soler, A.J., 2009. Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: electroejaculation and postmortem collection. Theriogenology 72, 160–168.
Giritharan, G., Ramakrishnappa, N., Balendran, A., Cheng, K.M., Rajamahendran, R., 2005. Development of in vitro tests to predict fertility of bulls. Can. J. Anim. Sci. 85, 47–52.
Gliedt, D.W., Rosenkrans Jr, C.F., Rorie, R.W., Rakes, J.M., 1996. Effects of oocyte maturation length, sperm capacitation time, and heparin on bovine embryo development. J. Dairy Sci. 79, 532–535.
Herrera, J., Fierro, R., Zayas, H., Conejo, J., Jiménez, I., García, A., Betancourt, M., 2002. Acrosome reaction in fertile and subfertile boar sperm. Syst. Biol. Reprod. Med. 48, 133–139.
Hochi, S., Korosue, K., Choi, Y.H., Oguri, N., 1996. In vitro capacitation of stallion spermatozoa assessed by the lysophosphatidylcholine-induced acrosome reaction and the penetration rate into in vitro-matured, zona-free mare oocytes. J. Equine Vet. Sci. 16, 244–248.
Hoflack, G., Van Soom, A., Maes, D., de Kruif, A., Opsomer, G., Duchateau, L., 2006. Breeding soundness and libido examination of Belgian Blue and Holstein Friesian artificial insemination bulls in Belgium and The Netherlands. Theriogenology 66, 207–216.
Januskauskas, A., Gil, J., Söderquist, L., Rodriguez-Martinez, H., 2000. Relationship between sperm response to glycosaminoglycans in vitro and non-return rates of Swedish dairy AI bulls. Reprod. Domest. Anim. 35, 207–212.
Januskauskas, A., Söderquist, L., Håård, M.G., Håård, M.C., Lundeheim, N., Rodriguez-Martinez, H., 1996. Influence of sperm number per straw on the post-thaw sperm viability and fertility of Swedish Red and White A.I. Bulls. Acta Vet. Scand. 37, 461–470.
Katska, L., Smorag, Z., 1996. Bull effect on in vitro embryo production in cattle. J. Physiol. Pharmacol. 47, 71–78.
Kharche, S.D., Goel, A.K., Jindal, S.K., Yadav, E.N., Yadav, P., Sinha, R., Sinha, N.K., 2009. Effect of serum albumin supplementation on in vitro capacitation and fertilization of caprine oocytes. Small Ruminant Res. 81, 85–89.
Kuroda, K., Fukushima, M., Harayama, H., 2007. Premature capacitation of frozen-thawed spermatozoa from subfertile Japanese Black cattle. J. Reprod. Dev. 53, 1079–1086.
Lessard, C., Siqueira, L.G., D'Amours, O., Sullivan, R., Leclerc, P., Palmer, C., 2011. Infertility in a beef bull due to a failure in the capacitation process. Theriogenology 76, 891–899.
Meyers, S.A., Liu, I.K.M., Overstreet, J.W., Vadas, S., Drobnis, E.Z., 1996. Zona pellucida binding and zona-induced acrosome reactions in horse spermatozoa: comparisons between fertile and subfertile stallions. Theriogenology 46, 1277–1288.
Miller, D.J., Winer, M.A., Ax, R.L., 1990. Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biol. Reprod. 42, 899–915.
Mirkena, T., Duguma, G., Haile, A., Tibbo, M., Okeyo, A.M., Wurzinger, M., Sölkner, J., 2010. Genetics of adaptation in domestic farm animals: a review. Livest. Sci. 132, 1–12.
Molnarova, Z., Machatkova, M., Machal, L., Horakova, J., Hanzalova, K., 2006. A potential relationship between the acrosome response characteristics of bovine spermatozoa and their in vitro fertilizing ability. Zygote 14, 63–69.
Mukhopadhyay, C.S., Verma, A., Joshi, B.K., Singh, A., Chakravarty, A.K., Dubey, P.P., 2008. In vitro acrosome reaction: its pertinence in assessing fertility of cryopreserved semen. Indian J. Anim. Res. 42, 201–204.
Parrish, J.J., Susko-Parrish, J., Winer, M.A., First, N.L., 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38, 1171–1180.
Parrish, J.J., Susko-Parrish, J.L., Graham, J.K., 1999. In vitro capacitation of bovine spermatozoa: role of intracellular calcium. Theriogenology 51, 461–472.
Pavlok, A., Kubelka, M., Pěknicová, J., 2001. The effect of various capacitation active compounds and capacitation time on the in vitro fertility and protein tyrosine phosphorylation profiles of bovine sperm. Zygote 9, 25–38.

Pontes, J.H.F., Silva, K.C.F., Basso, A.C., Rigo, A.G., Ferreira, C.R., Santos, G.M.G., Sanches, B.V., Porcionato, J.P.F., Vieira, P.H.S., Faifer, F.S., Sterza, F.A.M., Schenk, J.L., Seneda, M.M., 2010. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. Theriogenology 74, 1349–1355.

Rodero Serrano, E., Valera Córdoba, M. (Eds.), 2008. La Ganadería Andaluza en el siglo, vol. XXI., Junta de Andalucía, Sevilla.

Rodriguez, P.C., Satorre, M.M., Beconi, M.T., 2012. Effect of two intracellular calcium modulators on sperm motility and heparin-induced capacitation in cryopreserved bovine spermatozoa. Anim. Reprod. Sci. 131, 135–142.

Shamsuddin, M., Rodriguez-Martinez, H., Larsson, B., 1993. Fertilizing capacity of bovine spermatozoa selected after swim-up in hyaluronic acid-containing medium. Reprod. Fertil. Dev. 5, 307–315.

Sharma, R.K., Seifarth, K., Garlak, D., Agarwal, A., 1999. Comparison of three sperm preparation media. Int. J. Fertil. Women's Med. 44, 163–167.

Smetanina, I.G., Tatarinova, L.V., Krivokharchenko, A.S., 2006. In vitro fertilization of bovine oocytes in protein-free culture system. Russ. J. Dev. Biol. 37, 366–370.

Söderquist, L., Janson, L., Håård, M., Einarsson, S., 1996. Influence of season, age, breed and some other factors on the variation in sperm morphological abnormalities in Swedish dairy A.I. bulls. Anim. Reprod. Sci. 44, 91–98.

Solti, L., Crichton, E.G., Loskutoff, N.M., Cseh, S., 2000. Economical and ecological importance of indigenous livestock and the application of assisted reroduction to their preservation. Theriogenology 53, 149–162.

Somfai, T., Bodó, S., Nagy, S., Papp, Á.B., Iváncsics, J., Baranyai, B., Gócza, E., Kovács, A., 2002. Effect of swim up and Percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. Reprod. Domest. Anim. 37, 285–290.

Teixeira, H.C.A., Nascimento, N.V., McManus, C., Egito, A.A., Mariante, A.D.S., Ramos, A.F., 2011. Seasonal influence on semen traits and freezability from locally adapted Curraleiro bulls. Anim. Reprod. Sci. 125, 56–61.

Thundathil, J., Gil, J., Januskauskas, A., Larsson, B., Soderquist, L., Mapletoft, R., Rodriguez-Martinez, H., 1999. Relationship between the proportion of capacitated spermatozoa present in frozen–thawed bull semen and fertility with artificial insemination. Int. J. Androl. 22, 366–373.

Vadnais, M.L., Kirkwood, R.N., Specher, D.J., Chou, K., 2005. Effects of extender, incubation temperature, and added seminal plasma on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. Anim. Reprod. Sci. 90, 347–354.

Visconti, P.E., Kopf, G.S., 1998. Regulation of protein phosphorylation during sperm capacitation. Biol. Reprod. 59, 1–6.

Waberski, D., Petrounkina, A., Weitze, K.F., Töpfer-Petersen, E., 1999. In Vitro Assessment of Semen for the Prediction of Fertility. vol. 27, pp. 1–7.

Ward, F., Enright, B., Rizos, D., Boland, M., Lonergan, P., 2002. Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. Theriogenology 57, 2105–2117.

Ward, F., Rizos, D., Corridan, D., Quinn, K., Boland, M., Lonergan, P., 2001. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo. Mol. Reprod. Dev. 60, 47–55.

Whitfield, C.H., Parkinson, T.J., 1992. Relationship between fertility of bovine semen and in vitro induction of acrosome reactions by heparin. Theriogenology 38, 11–20.

Capítulo Tres / Chapter Three

CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD12140

Effects of oocyte quality, incubation time and maturation environment on the number of chromosomal abnormalities in IVF-derived early bovine embryos

Sebastian Demyda-Peyrás[A,D], *Jesus Dorado*[B], *Manuel Hidalgo*[B], *Jaouad Anter*[A], *Leonardo De Luca*[C], *Enrique Genero*[C] *and Miguel Moreno-Millán*[A]

[A]Department of Genetics, University of Cordoba, CN IV KM 396, Campus Rabanales CP 14071, Gregor Mendel Building, Cordoba, Spain.
[B]Animal Reproduction Group, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, CN IV KM 396, Campus Rabanales CP 14071, Spain.
[C]Animal Production Department, Lomas de Zamora University, Ruta 4 Km 2 CP 1834, Llavallol, Bs As, Argentina.
[D]Corresponding author. Email: ge2depes@uco.es

Abstract. Chromosomal aberrations are one of the major causes of embryo developmental failures in mammals. The occurrence of these types of abnormalities is higher in *in vitro*-produced (IVP) embryos. The aim of the present study was to investigate the effect of oocyte morphology and maturation conditions on the rate of chromosomal abnormalities in bovine preimplantational embryos. To this end, 790 early cattle embryos derived from oocytes with different morphologies and matured under different conditions, including maturation period (24 *v.* 36 h) and maturation media (five different serum supplements in TCM-199), were evaluated cytogenetically in three sequential experiments. The rates of normal diploidy and abnormal haploidy, polyploidy and aneuploidy were determined in each embryo. Throughout all the experiments, the rate of chromosomal abnormalities was significantly ($P < 0.05$) affected by oocyte morphology and maturation conditions (maturation time and culture medium). Lower morphological quality was associated with a high rate of chromosome abnormalities ($P < 0.05$). Moreover, polyploidy was associated with increased maturation time ($P < 0.01$), whereas the maturation medium significantly ($P < 0.05$) affected the rates of haploidy and polyploidy. In general, supplementing the maturation medium with oestrous cow serum or fetal calf serum resulted in higher rates of chromosomal aberrations ($P < 0.05$) compared with the other serum supplements tested (bovine steer serum, anoestroues cow serum, bovine amniotic fluid and bovine serum albumin). On the basis of the results of the present study, we conclude that the morphological quality of oocytes and the maturation conditions affect the rate of chromosomal abnormalities in IVP bovine embryos.

Additional keywords: aneuploidy, cattle, chromosome analysis, developmental failures, *in vitro* produced (IVP) embryos, maturation time, polyploidy, serum supplementation.

Received 1 May 2012, accepted 27 September 2012, published online 27 November 2012

Introduction

The past decade has seen constant improvements in the bovine IVF industry. At present, in some countries the number of *in vivo* and *in vitro*-produced (IVP) embryos is similar (Pontes *et al.* 2010). However, despite the considerable research undertaken in recent years, the overall efficiency of the IVP of cattle embryos remains low: only 30% of cultured oocytes reach the blastocyst stage (Lonergan 2007). It is well known that *in vivo*-derived embryos have a higher quality and viability to term than those produced by IVM, IVF and culture (Ulloa Ulloa *et al.* 2008*b*), even when the same donor cow is used (Pontes *et al.* 2009). Although the causes of this inefficiency are likely to be numerous and complex, the morphological quality of the oocyte at the beginning of the IVP process and the oocyte maturation protocols have been reported as major contributing factors. In this sense, it has been demonstrated that oocytes with a higher morphological quality (Class A and B) exhibit improved developmental rates to term in the subsequently formed

embryos (Hawk and Wall 1994). Furthermore, maturation media (Bilodeau-Goeseels 2006; Răty *et al.* 2011), maturation protocols (Yang *et al.* 1993) and serum supplementation (Fukui and Ono 1989; Pereira *et al.* 2010) have also been reported as potential causes of differences between *in vivo*-produced and IVP embryos. Thus, different *in vitro* oocyte maturation media, maturation protocols and media supplements are constantly being developed and tested to better meet the oocyte metabolic requirements as close to the ideal *in vivo* situation (Rizos *et al.* 2002; Purpera *et al.* 2009).

Many factors have been reported as causes of the increased developmental failure of IVP embryos (Van Soom *et al.* 2007). Of these, chromosomal abnormalities are thought to be a major contributor to developmental failure, having been associated with reduced developmental potential (King *et al.* 2006), impaired embryo viability (Kawarsky *et al.* 1996) and embryonic death and abortion (King 1990). In cattle, it has been reported that IVP embryos have a higher rate of chromosome abnormalities than their *in vivo* counterparts (Viuff *et al.* 1999). Similar results have been reported for the pig (Ulloa Ulloa *et al.* 2008*a*), horse (Rambags *et al.* 2005), goat (Villamediana *et al.* 2001) and sheep (Alexander *et al.* 2006). It has been demonstrated that the percentage of chromosomal abnormalities appears to be significantly influenced by the maturation environment of the oocytes (A'arabi *et al.* 1997), as well as post-fertilisation culture conditions for IVP embryos (Lonergan *et al.* 2004).

Currently, it is not possible to evaluate the chromosomal complement of matured oocytes prior to fertilisation in the IVF laboratory by simple and non-invasive techniques (Coticchio *et al.* 2004). Therefore, oocytes with normal maturation parameters can have an abnormal chromosomal composition, producing higher rates of fertilisation failures and development impairment. Despite the potential economic impact on livestock production, only a few cytogenetic studies of IVP embryos have been performed (Viuff *et al.* 1999; Jakobsen *et al.* 2006; Ulloa Ulloa *et al.* 2008; Garcia-Herreros *et al.* 2010). Moreover, these studies did not investigate the contribution of oocyte quality and maturation environment to these alterations.

Therefore, the aim of the present study was to examine the effects of oocyte morphological quality, oocyte maturation time, and oocyte maturation medium (serum supplementation) on the incidence of chromosomal abnormalities in IVP early bovine embryos.

Materials and methods

Media and reagents

Unless indicated otherwise, chemicals were purchased from Sigma-Aldrich (Madrid, Spain). Percoll was purchased from GE Healthcare (Madrid, Spain), Nunclon plastic dishes were obtained from Thermo Fisher Scientific (Madrid, Spain) and sterilisation filters used for the culture media were purchased from Millipore (Madrid, Spain). Oocytes and embryos were manipulated with 20-μL micropipette tips purchased from Eppendorf Iberica (Madrid, Spain).

Oestrous cow serum (ECS) was obtained as described previously (Ocaña-Quero *et al.* 1999*b*). Briefly, sterile complete blood samples were collected by jugular venipuncture of six oestrous cows. Serum was obtained by centrifuging pooled blood samples at 200*g* for 15 min at room temperature (RT), followed by heat inactivation (56°C, 30 min) and sterilisation through a 0.22-μm membrane filter. Serum aliquots (1 mL) were frozen and stored at −20°C until use. Anoestrous cow serum (ACS) was obtained using the same protocol following the collection of blood samples from six anoestrous cows.

In vitro *embryo production*

Bovine embryos were produced as described previously (Ocaña-Quero *et al.* 1999*a*), with some modifications, as detailed below.

Collection of ovaries and oocytes and IVM

Ovaries were collected from cows that had been killed in a local abattoir and were transported to the laboratory within 2 h of collection. The ovaries were washed three times in warm physiological saline solution (38.5°C) supplemented with kanamycin (25 mg mL^{-1}). Cumulus–oocyte complexes (COCs) were obtained from follicles that were 4–8 mm in diameter by aspiration with an 18-gauge needle and placed into a 15-mL conical tube with 2 mL warm H-TALP medium (38.5°C; Parrish *et al.* 1986). After 1 h sedimentation at 38.5°C, oocytes were poured on Petri dishes and examined under a stereomicroscope with a warm plate (38.5°C). In Experiment 1, oocytes were classified according to their morphology into one of four groups (Class A, B, C or D) according to Cetica *et al.* (1999) and Hawk and Wall (1994). Based on the outcome of Experiment 1, and to eliminate the possible detrimental effect of morphological quality, only Class A oocytes (compact, intact cumulus cell layers and good morphology) were used for Experiments 2 and 3. Selected COCs were washed twice in H-TALP and cultured for 24 h at 38.5°C in 5% CO_2 in groups of 100 in 500 μL modified bicarbonate-buffered TCM-199 medium supplemented with 10% fetal calf serum (FCS), 0.4 mM glutamine, 0.2 mM sodium pyruvate, 25 μg mL^{-1} FSH, 6.25 μg mL^{-1} LH, 2 μg mL^{-1} oestradiol and 50 mg mL^{-1} gentamicin.

Sperm capacitation and IVF

Frozen semen straws from different bulls of the Retinta breed, with a history of proven fertility, were thawed in a water bath at 38°C for 1 min. Viable spermatozoa were selected by centrifugation through a discontinuous Percoll gradient (45% and 90% (v/v) in Sp-TALP; Parrish *et al.* 1986) and washed twice in warm equilibrated Sp-TALP medium (38.5°C). Spermatozoa were adjusted to a final concentration of 1×10^6 spermatozoa mL^{-1} using IVF-TALP (Parrish *et al.* 1986), supplemented with 6 mg mL^{-1} bovine serum albumin (BSA) and 100 mg mL^{-1} heparin. Groups of 60 oocytes were washed in 500 μL equilibrated IVF-TALP and incubated with spermatozoa for 20–24 h at 38.5°C in 5% CO_2.

Culture of zygotes

After 20–24 h incubation, groups of 100 presumptive zygotes were denuded by vortexing in 15-mL tubes for 3 min.

Thereafter, they were transferred to synthetic oviducal fluid (SOF), supplemented with BME 50X amino acid solution (B6766, Sigma Aldrich), MEM 100X non-essential amino acid solution (M7145, Sigma Aldrich) and 5% (v/v) FCS, and cultured in 50-μL droplets under mineral oil in groups of 25 for 72 h at 38.5°C in 5% CO_2.

Chromosome preparation

Chromosome preparations were established according to our standard method (Ocaña-Quero *et al.* 1999*a*). At the end of the culture period, colchicine (0.1 μg mL^{-1}) was added to the culture medium and embryos were cultured for up to a further 6 h. The embryos were then placed in 500 μL hypotonic solution of 0.88% (w/v) tri-sodium citrate for 45–60 min. Thereafter, embryos were fixed in approximately 500 μL Carnoy's 1 : 1 fixative (one part acetic acid : one part methanol) for 10 min before being placed in 3 : 1 Carnoy's fixative overnight. Each embryo was then fixed onto a clean glass slide and the blastomeres were separated using acetic acid (100%, v/v). Each nucleus was spread by dropping several drops of Carnoy's 1 : 1 fixative. Samples were air dried and stained with 5% Giemsa solution for 10 min. Only those blastomeres containing an analysable metaphase (intact and non-overlapping chromosomes) were assessed at ×1250 magnification under an optical microscope (Reichert-Jung Polyvar, Austria).

Cytogenetic criteria

Only embryos with more than one analysable metaphase were included in the present study. Embryos with 60 chromosomes in all countable metaphases were defined as normal diploid ($2n = 60$). Those in which all analysable metaphases contained 30 chromosomes were defined as haploid. If the embryo had more than two sets of chromosomes ($3n = 90$ or $4n = 120$) in all blastomeres, it was defined as polyploid. Finally, those embryos with an abnormal chromosomal number were defined as aneuploid.

Experimental design

Experiment 1

The aim of this experiment was to determine the effect of morphological oocyte quality on the appearance of chromosomal abnormalities in early IVP bovine embryos. In all, 249 oocytes were classified into of four groups (Class A, B, C or D) on the basis of a subjective assessment of their quality, as described previously by Hawk and Wall (1994). Oocytes were fertilised, cultured and analysed cytogenetically as described above.

Experiment 2

The aim of this experiment was performed to determine the effect of oocyte maturation time on the appearance of chromosomal abnormalities in early IVP bovine embryos. In this experiment, 157 embryos derived from Class A oocytes identified in Experiment 1 were matured for either 24 or 36 h. Thereafter, oocytes were fertilised, cultured and analysed cytogenetically as described above.

Experiment 3

This experiment was performed to determine the effect of different serum supplementation strategies during *in vitro* oocyte maturation on the percentage of chromosomal abnormalities observed in IVP early bovine embryos. In this experiment, 384 embryos derived from Class A oocytes were produced as described previously. Oocytes were matured in one of six groups, consisting of TCM-199 medium supplemented with: (1) 10% ECS ($n = 62$); (2) 10% FCS ($n = 59$; F2442; Sigma Aldrich); (3) 10% ACS ($n = 59$); (4) 10% bovine amniotic fluid (BAF; $n = 60$; A5130; Sigma Aldrich); (5) 0.6% (w/v) BSA ($n = 74$); and (6) 10% bovine steer serum (BSS; $n = 70$; A9433, Sigma Aldrich), used as a control. After maturation, oocytes were fertilised, cultured for a period of 72 h and analysed cytogenetically as described above.

Statistical analysis

Data were analysed by the Chi-squared test using SAS software (SAS Institute, Cary, NC, USA). Differences among treatments were evaluated using a two-tailed Z score test. $P < 0.05$ was considered significant.

Results

Oocyte morphological quality, oocyte maturation time and serum supplementation were found to have significant ($P < 0.05$) effects on the percentage of abnormal chromosomal complements across all embryos analysed.

Experiment 1

There was a significant ($P < 0.05$) relationship between oocyte quality and the ploidy of the resulting embryos (Table 1). The number of normal diploid embryos increased significantly ($P < 0.05$) with increasing morphological quality of the oocyte. Therefore, the lowest percentage of total chromosomal abnormalities ($P < 0.05$) was observed only in embryos derived from oocytes of superior quality (Class A). When each type of chromosomal abnormality was analysed separately, the highest percentage of haploidy ($P < 0.05$) was observed in embryos derived from Class C and D oocytes. Similarly, the percentage of polyploid and aneuploid embryos was significantly ($P < 0.05$) lower for those derived from Class A oocytes compared with those from Class B, C and D oocytes.

Experiment 2

Chromosomal abnormality rates were significantly ($P < 0.01$) higher in oocytes matured for 36 h than in those matured for 24 h (Table 2). Interestingly, only polyploidy rates differed significantly ($P < 0.01$) between treatments and no significant ($P > 0.05$) differences were found between the rates of the other types of chromosomal abnormalities (i.e. haploid and aneuploid) and oocytes matured for different times (24 vs 36 h).

Experiment 3

Supplementation of the maturation medium with different sera had a significant ($P < 0.05$) effect on the incidence of chromosomal abnormalities of IVP bovine embryos (Table 3).

Table 1. Analysis of the chromosomal complement of Day 3 *in vitro*-produced bovine embryos derived from oocytes classified on the basis of their morphological quality

Within a row, values with different superscript letters differ significantly ($P < 0.05$, two-tailed Z-score test)

Oocyte quality	Total no. embryos analysed	No. normal diploid embryos (%)	No. abnormal embryos (%)			
			Total	Haploid	Polyploid	Aneuploid
Class A	60	56^{a} (93.33)	4^{c} (6.67)	1^{bc} (1.67)	2^{b} (3.33)	1^{b} (1.67)
Class B	65	53^{ab} (81.54)	12^{b} (18.46)	2^{b} (3.08)	6^{ab} (9.23)	4^{ab} (6.15)
Class C	61	45^{bc} (73.77)	6^{ab} (26.23)	6^{ab} (9.84)	6^{ab} (9.84)	4^{ab} (6.56)
Class D	63	39^{c} (61.90)	24^{a} (38.10)	10^{a} (15.87)	8^{a} (12.70)	6^{a} (9.52)

Table 2. Analysis of the chromosomal complement of Day 3 *in vitro*-produced bovine embryos derived from oocytes matured for either 24 or 36 h

Within a row, values with different uppercase superscript letters differ significantly ($P < 0.01$, expected proportion test); values with different lowercase superscript letters differ significantly ($P < 0.05$, two-tailed Z-score test)

Duration of oocyte maturation	No. embryos analysed	No. normal diploid embryos (%)	No. abnormal embryos (%)			
			Total	Haploid	Polyploid	Aneuploid
24 h	77	71^{A} (92.21)	6^{B} (7.79)	2^{a} (2.60)	3^{A} (3.90)	1^{a} (1.30)
36 h	80	60^{B} (75.00)	20^{A} (25.00)	6^{a} (7.50)	12^{B} (15.00)	2^{a} (2.50)

Table 3. Analysis of the chromosomal complement of Day 3 *in vitro*-produced bovine embryos derived from oocytes matured in different culture media

Within a row, values with different superscript letters differ significantly ($P < 0.05$, two-tailed Z-score test). BSS, bovine steer serum; ACS, anoestrous cow serum; BAF, bovine amniotic fluid; BSA, bovine serum albumin; FCS, fetal calf serum; ECS, oestrous cow serum

Culture medium supplement	No. embryos analysed	No. normal diploid embryos (%)	No. abnormal embryos (%)			
			Total	Haploid	Polyploid	Aneuploid
BSS	70	66^{a} (94.29)	4^{c} (5.71)	2^{b} (2.86)	1^{b} (1.43)	1^{a} (1.43)
ACS	59	54^{a} (91.53)	5^{c} (8.47)	1^{b} (1.69)	3^{b} (5.08)	1^{a} (1.69)
BAF	60	54^{abc} (90.00)	6^{bc} (10.00)	2^{b} (3.33)	2^{b} (3.33)	2^{a} (3.33)
BSA	74	66^{ab} (89.19)	8^{bc} (10.81)	5^{ab} (6.76)	3^{b} (4.05)	0^{a} (0.00)
FCS	59	46^{bcd} (77.97)	13^{b} (22.03)	5^{ab} (8.47)	6^{ab} (10.17)	2^{a} (3.39)
ECS	62	37^{d} (59.68)	25^{a} (40.32)	11^{a} (17.74)	12^{a} (19.35)	2^{a} (3.23)

In general, the use of ECS or FCS resulted in higher rates of chromosomal abnormalities ($P < 0.05$) compared with the other serum supplements. Moreover, the rates of haploidy and polyploidy were significantly ($P < 0.05$) higher in embryos matured in the presence of ECS. However, there were no significant ($P > 0.05$) differences in the rate of aneuploidy between the different supplement groups.

Discussion

Chromosomal abnormalities have been suggested as a major cause of failure of IVP embryos (King *et al.* 2006). It appears that the incidence of these abnormalities is influenced by the method of embryo handling used during these procedures, as well as the suboptimal culture environment in which the embryos must develop (King *et al.* 2006). In the present study, we evaluated the effects of oocyte quality and their IVM environment on the chromosomal make-up of the subsequent embryos. The results indicate that an initial poor oocyte quality, as well as suboptimal maturation conditions, result in a higher rate of chromosome abnormalities at the earliest stages of embryonic development.

Experiment 1 in the present study was undertaken to determine the importance of oocyte morphology in the incidence of chromosomal abnormalities in subsequent embryos. It has been reported previously that the initial oocyte quality has a considerable impact on early embryonic survival, as well as the establishment and maintenance of pregnancy (Krisher 2004). In the present study, the rate of chromosomal abnormalities in embryos was significantly affected by the morphological quality of the oocytes. Other studies have also reported that maturation rates and embryo development are strongly related to the

original morphology of the oocyte (Long *et al.* 1994; Blondin and Sirard 1995). However, to our knowledge, no previous studies have evaluated the chromosomal composition of embryos derived from specific morphological classifications of oocytes as possible cause of the losses seen for IVP cattle embryos. In the present study, higher rates of chromosomal abnormalities were observed in low-quality oocytes that begin to multiply after fertilisation (i.e. those with a pale and heterogeneous cytoplasm; Class D). This may be due to the fact that these types of oocytes have a low density of organelles to support the meiotic process, which leads to higher rates of meiotic failure (Nagano *et al.* 2006). Conversely, the lower incidence of morphologically normal spindles in the low-quality oocytes results in higher rates of chromosomal gains or losses during replication (Long *et al.* 1994). It has been reported that the pregnancy rate associated with embryos derived from lower-grade oocytes that have poor cytoplasmic quality (granularity or discolouration of the cytoplasm, vacuolisation and incorporations such as refractile bodies) is fivefold lower than that of embryos derived from oocytes classified as being of 'normal' quality (Ebner *et al.* 2003). It is of note that, in the present study, high-quality oocytes (Class A) exhibited a lower polyploidy rate. Recent studies have shown that the incidence of polyspermic fertilisation, a major cause of embryo polyploidy, is higher in low-quality oocytes compared with those with optimal morphology (Wortzman and Evans 2005). This may be due to delayed and incomplete exocytosis of the cortical granules (Wang *et al.* 1997) or to premature release of cortical granules from low-quality oocytes (Suzuki *et al.* 2003), causing a weaker block to polyspermy.

In Experiment 2, embryos derived from high-quality oocytes (Class A) were matured under different conditions (24 or 36 h) before being cultured *in vitro*. A higher rate of chromosomal alterations was observed when oocytes were matured for 36 h. Longer maturation times are known to decrease the percentage of embryos that reach the blastocyst stage (Gliedt *et al.* 1996) and stimulate higher nuclear maturation rates via greater positive feedback from cumulus cells (Barrett and Albertini 2010). However, over-maturation for >24 h promotes an 'aging' effect, with signs of degeneration probably due to the excessive time in spent a suboptimal maturation environment (Máximo *et al.* 2012). Of note, in the present study only rates of polyploidy were significantly higher when the maturation time was increased. This abnormality has been noted as a major cause of developmental failure for IVP embryos in mammals. Specifically, it has been suggested that maturation prepares the oocyte to interact with the fertilising spermatozoon at a particular moment in time (Dale and Defelice 2011) and, if the oocyte is not fertilised, the maturation processes continue and the cell ages, resulting in higher polyspermy rates (Tarín 1996). In a later study, Tarín *et al.* (1999) suggested that post-ovulation aging of oocytes may lead to a series of ultrastructural changes related to the integrity of the meiotic spindle. Moreover, Tarín *et al.* (1999) suggest that these changes may result in higher rates of failure of extrusion of the second polar body by the oocyte after sperm penetration, leading to higher polyploidy rates. Polyspermy is of particular concern, particularly in pig embryos (Niwa 1993). In the porcine, partial acrosome reaction induced by IVF medium and sperm fertilisation doses have been reported as the primary causes of polyspermy (Funahashi 2003).

Previous studies have demonstrated the effects of the culture system on the ploidy of IVM oocytes in humans (Christopikou *et al.* 2010) and livestock (Lechniak *et al.* 2005; Ueno *et al.* 2005; Ocaña-Quero *et al.* 1999*b*). Furthermore, the effects of the culture system on oocyte nuclear maturation rates and the blastocyst yield of IVF-derived embryos have been reported for goats (Tajik and Esfandabadi 2003), pigs (Ott *et al.* 2002) and cattle (Russell *et al.* 2006; Ocaña-Quero *et al.* 1999*b*). A possible explanation for the effects of the culture conditions may involve triggering of age-related changes in oocytes due to oxidative damage associated with high cellular metabolism and genomic instability (e.g. mitochondrial mutations and telomere shortening) (Pan *et al.* 2008). This is most likely due to decreased energy supply from oxidative phosphorylation in the mitochondria within the oocyte (Eichenlaub-Ritter *et al.* 2011). The results of the present study are in agreement with theses hypothesis, because we found a clear effect of the culture medium on the ploidy of the embryos. However, it has been reported that mature oocytes appear fairly normal until the resumption of maturation, when they form aberrant spindles (Ebner *et al.* 2003). Because we cannot evaluate the chromosomal composition and meiotic spindle prior to fertilisation, it can be assumed that oocytes with a higher level of chromosomal abnormalities as a result of the suboptimal environmental conditions (A'arabi *et al.* 1997) will produce embryos with a higher rate of abnormalities that may actually exhibit normal development at the very early stages (King *et al.* 2006).

In Experiment 3, higher rates of chromosomal abnormalities were observed in the culture medium supplemented with ECS. It has been reported that the use of ECS results in higher FSH, LH and oestrogen levels in the maturation medium (Ott *et al.* 2002). These hormones have been associated with oocyte aging (Liu *et al.* 2011), marked ultrastructural changes to embryonic cells and higher rates of blastomeric apoptosis (Ott *et al.* 2002) and chromosomal abnormalities (Vialard *et al.* 2011). Indeed, embryos derived from these oocytes can develop aberrant mitotic spindles, leading to a failure of chromatid migration to the cell poles during cell division, DNA replication without associated cytokinesis and, consequently, to an altered ploidy (Liu and Keefe 2002).

The lack of significant differences in aneuploidy rates in Experiments 2 and 3 suggests that the maturation environment (oocyte maturation time and supplementation of the culture medium) do not have that great an impact on the appearance of these types of chromosomal abnormalities. In fact, aneuploidy has been reported to be related mainly to intrinsic factors in human (Frumkin *et al.* 2008) and bovine (Lechniak *et al.* 1996; Nicodemo *et al.* 2010) oocytes.

Previous studies have suggested that the post-fertilisation culture environment of the developing embryo can affect the incidence and severity of chromosomal abnormalities in the resulting blastocyst (Lonergan *et al.* 2004; Ulloa Ulloa *et al.* 2008*b*; Xu *et al.* 2008). However, all these studies evaluated the chromosomal complement of embryos at the blastocyst stage. Dieleman *et al.* (2002) suggest that the ploidy of the resulting embryos is less affected by the culture environment at the early

stages of development. It has been reported previously that long-term incubation induces developmental failures in embryos as a result of their exposure to higher levels of toxic metabolites and oxidative stress at later stages of development in culture (Tajik *et al.* 2008; Mermillod *et al.* 2010).

In conclusion, both the morphological quality of oocytes and the maturation protocols affect the rate of chromosomal abnormalities in IVP cattle embryos. Further studies are necessary to improve the oocyte maturation protocols used in the *in vitro* cattle embryo production industry to minimise the appearance of chromosomal abnormalities in subsequent embryos.

Acknowledgements

The authors thank the COVAP slaughterhouse (Pozoblanco, Spain) for providing the ovaries used in the present study. The authors also thank to Dr Ann Van Soom, Ms Petra Van Damme and Ms Isabel Lemahieu for their technical assistance. SD was supported by a grant from MAEC (Ministerio de Asuntos Exteriores y Cultura) – AECID (Agencia Española de Cooperacion Internacional y Desarrollo) (Spain). This study was supported by the Molecular and Applied Animal Cytogenetic Lab, University of Cordoba, Spain.

References

A'arabi, S. Y., Roussel, J. D., and Chandler, J. E. (1997). Chromosomal analysis of mammalian oocytes matured *in vitro* with various culture systems. *Theriogenology* **48**, 1173–1183. doi:10.1016/S0093-691X(97)00350-6

Alexander, B., Coppola, G., Berardina, D., Rho, G.J., St John, E., Bett, D. H., and King, W. A. (2006). The effect of 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX) on the development and chromosomal complement of sheep parthenogenetic and nuclear transfer embryos. *Mol. Reprod. Dev.* **73**, 20–30. doi:10.1002/MRD.20372

Barrett, S. L., and Albertini, D. F. (2010). Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence. *J. Assist. Reprod. Genet.* **27**, 29–39. doi:10.1007/S10815-009-9376-9

Bilodeau-Goeseels, S. (2006). Effects of culture media and energy sources on the inhibition of nuclear maturation in bovine oocytes. *Theriogenology* **66**, 297–306. doi:10.1016/J.THERIOGENOLOGY.2005.11.014

Blondin, P., and Sirard, M. A. (1995). Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol. Reprod. Dev.* **41**, 54–62. doi:10.1002/MRD.1080410109

Cetica, P. D., Dalvit, G. C., and Beconi, M. T. (1999). Study of evaluation criteria used for *in vitro* bovine oocyte selection and maturation. *Biocell* **23**, 125–133.

Christopikou, D., Karamalegos, C., Doriza, S., Argyrou, M., Sisi, P., Davies, S., and Mastrominas, M. (2010). Spindle and chromosome configurations of human oocytes matured *in vitro* in two different culture media. *Reprod. Biomed. Online* **20**, 639–648. doi:10.1016/J.RBMO.2010.02.005

Coticchio, G., Sereni, E., Serrao, L., Mazzone, S., Iadarola, I., and Borini, A. (2004). What criteria for the definition of oocyte quality? *Ann. N. Y. Acad. Sci.* **1034**, 132–144. doi:10.1196/ANNALS.1335.016

Dale, B., and Defelice, L. (2011). Polyspermy prevention: facts and artifacts? *J. Assist. Reprod. Genet.* **28**, 199–207. doi:10.1007/S10815-010-9513-5

Dieleman, S. J., Hendriksen, P. J. M., Viuff, D., Thomsen, P. D., Hyttel, P., Knijn, H. M., Wrenzycki, C., Kruip, T. A. M., Niemann, H., Gadella, B. M., Bevers, M. M., and Vos, P. L. A. M. (2002). Effects of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology* **57**, 5–20. doi:10.1016/S0093-691X(01)00655-0

Ebner, T., Moser, M., Sommergruber, M., and Tews, G. (2003). Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review. *Hum. Reprod. Update* **9**, 251–262. doi:10.1093/HUMUPD/DMG021

Eichenlaub-Ritter, U., Wieczorek, M., Lüke, S., and Seidel, T. (2011). Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. *Mitochondrion* **11**, 783–796. doi:10.1016/J.MITO.2010.08.011

Frumkin, T., Malcov, M., Yaron, Y., and Ben-Yosef, D. (2008). Elucidating the origin of chromosomal aberrations in IVF embryos by preimplantation genetic analysis. *Mol. Cell. Endocrinol.* **282**, 112–119. doi:10.1016/J.MCE.2007.11.009

Fukui, Y., and Ono, H. (1989). Effects of sera, hormones and granulosa cells added to culture medium for *in-vitro* maturation, fertilization, cleavage and development of bovine oocytes. *J. Reprod. Fertil.* **86**, 501–506. doi:10.1530/JRF.0.0860501

Funahashi, H. (2003). Polyspermic penetration in porcine IVM–IVF systems. *Reprod. Fertil. Dev.* **15**, 167–177. doi:10.1071/RD02076

Garcia-Herreros, M., Carter, T. F., Villagmez, D. A. F., MacAulay, A. D., Rath, D., King, W. A., and Lonergan, P. (2010). Incidence of chromosomal abnormalities in bovine blastocysts derived from unsorted and sex-sorted spermatozoa. *Reprod. Fertil. Dev.* **22**, 1272–1278. doi:10.1071/RD10052

Gliedt, D. W., Rosenkrans, C. F., Jr, Rorie, R. W., and Rakes, J. M. (1996). Effects of oocyte maturation length, sperm capacitation time, and heparin on bovine embryo development. *J. Dairy Sci.* **79**, 532–535. doi:10.3168/JDS.S0022-0302(96)76396-8

Hawk, H. W., and Wall, R. J. (1994). Improved yields of bovine blastocysts from *in vitro*-produced oocytes. II. Media and co-culture cells. *Theriogenology* **41**, 1585–1594. doi:10.1016/0093-691X(94)90823-2

Jakobsen, A. S., Thomsen, P. D., and Avery, B. (2006). Few polyploid blastomeres in morphologically superior bovine embryos produced in vitro. *Theriogenology* **65**(4), 870–881. doi:10.1016/J.THERIOGENOLOGY.2005.06.014

Kawarsky, S. J., Basrur, P. K., Stubbings, R. B., Hansen, P. J., and King, W. A. (1996). Chromosomal abnormalities in bovine embryos and their influence on development. *Biol. Reprod.* **54**, 53–59. doi:10.1095/BIOLREPROD54.1.53

King, W. A. (1990). Chromosome abnormalities and pregnancy failure in domestic animals. *Adv. Vet. Sci. Comp. Med.* **34**, 229–250.

King, W. A., Coppola, G., Alexander, B., Mastromonaco, G., Perrault, S., Nino-Soto, M. I., Pinton, A., Joudrey, E. M., and Betts, D. H. (2006). The impact of chromosomal alteration on embryo development. *Theriogenology* **65**, 166–177. doi:10.1016/J.THERIOGENOLOGY.2005.09.031

Krisher, R. L. (2004). The effect of oocyte quality on development. *J. Anim. Sci.* **82**(Suppl. E), E14–E23.

Lechniak, D., Świtoński, M., and Sosnowski, M. (1996). The incidence of bovine diploid oocytes matured *in vitro*. *Theriogenology* **46**, 267–277. doi:10.1016/0093-691X(96)00183-5

Lechniak, D., Szczepankiewicz, D., Kauss, D., Szulc, J., and Szydlowski, M. (2005). IVM media, oocyte diameter and donor genotype at RYR1 locus in relation to the incidence of porcine diploid oocytes after maturation *in vitro*. *Theriogenology* **64**, 202–212. doi:10.1016/J.THERIOGENOLOGY.2004.11.019

Liu, L., and Keefe, D. L. (2002). Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice. *Hum. Reprod.* **17**, 2678–2685. doi:10.1093/HUMREP/17.10.2678

Liu, S., Feng, H. L., Marchesi, D., Chen, Z. J., and Hershlag, A. (2011). Dose-dependent effects of gonadotropin on oocyte developmental

competence and apoptosis. *Reprod. Fertil. Dev.* **23**, 990–996. doi:10.1071/RD11079

Lonergan, P. (2007). State-of-the-art embryo technologies in cattle. *Soc. Reprod. Fertil. Suppl.* **64**, 315–325.

Lonergan, P., Pedersen, H. G., Rizos, D., Greve, T., Thomsen, P. D., Fair, T., Evans, A., and Boland, M. P. (2004). Effect of the post-fertilization culture environment on the incidence of chromosome aberrations in bovine blastocysts. *Biol. Reprod.* **71**, 1096–1100. doi:10.1095/BIOLREPROD.104.030635

Long, C. R., Damiani, P., Pinto-Correia, C., MacLean, R. A., Duby, R. T., and Robl, J. M. (1994). Morphology and subsequent development in culture of bovine oocytes matured *in vitro* under various conditions of fertilization. *J. Reprod. Fertil.* **102**, 361–369. doi:10.1530/JRF.0.1020361

Máximo, D. M., Martins da Silva, I. G., Mondadori, R. G., Neves, J. P., and Lucci, C. M. (2012). Ultrastructural characteristics of sheep oocytes during *in vitro* maturation (IVM). *Small Rumin. Res.* **105**, 210–215. doi:10.1016/J.SMALLRUMRES.2011.11.021

Mermillod, P., Schmaltz, B., Perreau, C., Tsikis, G., Martinot, E., Cordova, A., and Locatelli, Y. (2010). Early development of ruminant embryos, autonomous process or the result of a positive dialog with surrounding maternal tissues? *Acta Sci. Vet.* **38**(Suppl. 2), S521–S533.

Nagano, M., Katagiri, S., and Takahashi, Y. (2006). Relationship between bovine oocyte morphology and *in vitro* developmental potential. *Zygote* **14**, 53–61. doi:10.1017/S0967199406003510

Nicodemo, D., Pauciullo, A., Cosenza, G., Peretti, V., Perucatti, A., Di Meo, G. P., Ramunno, L., Iannuzzi, L., Rubes, J., and Di Berardino, D. (2010). Frequency of aneuploidy in *in vitro*-matured MII oocytes and corresponding first polar bodies in two dairy cattle (*Bos taurus*) breeds as determined by dual-color fluorescent *in situ* hybridization. *Theriogenology* **73**, 523–529. doi:10.1016/J.THERIOGENOLOGY.2009.10.007

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

Ocaña-Quero, J. M., Pinedo-Merlin, M., and Moreno-Millan, M. (1999*a*). Cytogenetic study of *in vitro*-derived bovine embryos. *Vet. J.* **158**, 228–233. doi:10.1053/TVJL.1998.0330

Ocaña-Quero, J. M., Pinedo-Merlin, M., and Moreno-Millan, M. (1999*b*). Influence of follicle size, medium, temperature and time on the incidence of diploid bovine oocytes matured *in vitro*. *Theriogenology* **51**, 667–672. doi:10.1016/S0093-691X(99)00005-9

Ott, M., Schernthaner, W., Sinowatz, F., and Wolf, E. (2002). Effects of bovine serum albumin and estrous cow serum on development and ultrastructure of *in vitro*-produced porcine embryos. *Anat. Histol. Embryol.* **31**, 151–157. doi:10.1046/J.1439-0264.2002.00384.X

Pan, H., Ma, P., Zhu, W., and Schultz, R. M. (2008). Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Dev. Biol.* **316**, 397–407. doi:10.1016/J.YDBIO.2008.01.048

Parrish, J. J., Susko-Parrish, J. L., Critser, E. S., Eyestone, W. H., and First, N. L. (1986). Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology* **25**, 591–600. doi:10.1016/0093-691X(86)90143-3

Pereira, M. M., MacHado, M. A., Costa, F. Q., Serapiao, R. V., Viana, J. H. M., and Camargo, L. S. A. (2010). Effect of oxygen tension and serum during IVM on developmental competence of bovine oocytes. *Reprod. Fertil. Dev.* **22**, 1074–1082. doi:10.1071/RD10007

Pontes, J. H. F., Nonato-Junior, I., Sanches, B. V., Ereno-Junior, J. C., Uvo, S., Barreiros, T. R. R., Oliveira, J. A., Hasler, J. F., and Seneda, M. M. (2009). Comparison of embryo yield and pregnancy rate between *in vivo* and *in vitro* methods in the same Nelore (*Bos indicus*) donor cows. *Theriogenology* **71**, 690–697. doi:10.1016/J.THERIOGENOLOGY.2008.09.031

Pontes, J. H. F., Silva, K. C. F., Basso, A. C., Rigo, A. G., Ferreira, C. R., Santos, G. M. G., Sanches, B. V., Porcionato, J. P. F., Vieira, P. H. S., Faifer, F. S., Sterza, F. A. M., Schenk, J. L., and Seneda, M. M. (2010). Large-scale *in vitro* embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and *indicus–taurus* dairy cows using sexed sperm. *Theriogenology* **74**, 1349–1355. doi:10.1016/J.THERIOGENOLOGY.2010.06.004

Purpera, M. N., Giraldo, A. M., Ballard, C. B., Hylan, D., Godke, R. A., and Bondioli, K. R. (2009). Effects of culture medium and protein supplementation on mRNA expression of *in vitro* produced bovine embryos. *Mol. Reprod. Dev.* **76**, 783–793. doi:10.1002/MRD.21028

Rambags, B. P. B., Krijtenburg, P. J., Van Drie, H. F., Lazzari, G., Galli, C., Pearson, P. L., Colenbrander, B., and Stout, T. A. E. (2005). Numerical chromosomal abnormalities in equine embryos produced *in vivo* and *in vitro*. *Mol. Reprod. Dev.* **72**, 77–87. doi:10.1002/MRD.20302

Räty, M., Ketoja, E., Pitkänen, T., Ahola, V., Kananen, K., and Peippo, J. (2011). *In vitro* maturation supplements affect developmental competence of bovine cumulus–oocyte complexes and embryo quality after vitrification. *Cryobiology* **63**, 245–255. doi:10.1016/J.CRYOBIOL.2011.09.134

Rizos, D., Ward, F., Duffy, P., Boland, M. P., and Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**, 234–248. doi:10.1002/MRD.1153

Russell, D. F., Baqir, S., Bordignon, J., and Betts, D. H. (2006). The impact of oocyte maturation media on early bovine embryonic development. *Mol. Reprod. Dev.* **73**, 1255–1270. doi:10.1002/MRD.20553

Suzuki, H., Saito, Y., Kagawa, N., and Yang, X. (2003). *In vitro* fertilization and polyspermy in the pig: factors affecting fertilization rates and cytoskeletal reorganization of the oocyte. *Microsc. Res. Tech.* **61**, 327–334. doi:10.1002/JEMT.10345

Tajik, P., and Esfandabadi, N. S. (2003). *In vitro* maturation of caprine oocytes in different culture media. *Small Rumin. Res.* **47**, 155–158. doi:10.1016/S0921-4488(02)00238-9

Tajik, P., Beheshti-Govij, R., Soleimani-Rad, J., Ghasemzadeh-Nava, H., and Mohamadi-Roshandeh, A. (2008). Effects of different concentrations of bovine follicular fluid and estrous cow serum on development of murine 2-cell embryos. *Pak. J. Biol. Sci.* **11**, 628–632. doi:10.3923/PJBS.2008.628.632

Tarín, J. J. (1996). Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol. Hum. Reprod.* **2**, 717–724. doi:10.1093/MOLEHR/2.10.717

Tarín, J. J., Trounson, A. O., and Sathananthan, H. (1999). Origin and ploidy of multipronuclear zygotes. *Reprod. Fertil. Dev.* **11**, 273–279. doi:10.1071/RD99057

Ueno, S., Kurome, M., Ueda, H., Tomii, R., Hiruma, K., and Nagashima, H. (2005). Effects of maturation conditions on spindle morphology in porcine MII oocytes. *J. Reprod. Dev.* **51**, 405–410. doi:10.1262/JRD.16091

Ulloa Ulloa, C. M., Yoshizawa, M., Komoriya, E., Mitsui, A., Nagai, T., and Kikuchi, K. (2008*a*). The blastocyst production rate and incidence of chromosomal abnormalities by developmental stage in *in vitro* produced porcine embryos. *J. Reprod. Dev.* **54**, 22–29. doi:10.1262/JRD.19102

Ulloa Ulloa, C. M., Yoshizawa, M., Yamashita, A., Hama, S., Mitsui, A., Hashi, C., Abe, H., Hoshi, H., Fukui, E., and Matsumoto, H. (2008*b*). Blastocyst production from *in vitro*-produced day-2 bovine embryos classified by cleavage stage, and cytogenetical evaluation of the resultant day-8 blastocysts. *J. Reprod. Dev.* **54**, 465–472. doi:10.1262/JRD.20036

Van Soom, A., Vandaele, L., Goossens, K., de Kruif, A., and Peelman, L. (2007). Gamete origin in relation to early embryo development. *Theriogenology* **68**(Suppl. 1), S131–S137. doi:10.1016/J.THERIOGENOLOGY.2007.03.019

Vialard, F., Boitrelle, F., Molina-Gomes, D., and Selva, J. (2011). Predisposition to aneuploidy in the oocyte. *Cytogenet. Genome Res.* **133**, 127–135. doi:10.1159/000324231

Villamediana, P., Vidal, F., and Paramio, M. T. (2001). Cytogenetic analysis of caprine 2- to 4-cell embryos produced *in vitro*. *Zygote* **9**, 193–199. doi:10.1017/S0967199401001204

Viuff, D., Rickords, L., Offenberg, H., Hyttel, P., Avery, B., Greve, T., Olsaker, I., Williams, J. L., Callesen, H., and Thomsen, P. D. (1999). A high proportion of bovine blastocysts produced *in vitro* are mixoploid. *Biol. Reprod.* **60**, 1273–1278. doi:10.1095/BIOLREPROD60.6.1273

Wang, W. H., Hosoe, M., and Shioya, Y. (1997). Induction of cortical granule exocytosis of pig oocytes by spermatozoa during meiotic maturation. *J. Reprod. Fertil.* **109**, 247–255. doi:10.1530/JRF.0.1090247

Wortzman, G. B., and Evans, J. P. (2005). Membrane and cortical abnormalities in post-ovulatory aged eggs: analysis of fertilizability and establishment of the membrane block to polyspermy. *Mol. Hum. Reprod.* **11**, 1–9. doi:10.1093/MOLEHR/GAH125

Xu, L., Wang, Y., Zhou, P., Cao, Y. X., Huang, T. H., and Chian, R. C. (2008). Cytogenetic analysis of *in vivo* and *in vitro* matured oocytes derived from naturally cycling and stimulated mice. *Syst. Biol. Reprod. Med.* **54**, 155–162. doi:10.1080/19396360802102012

Yang, X., Jiang, S., and Foote, R. H. (1993). Bovine oocyte development following different oocyte maturation and sperm capacitation procedures. *Mol. Reprod. Dev.* **34**, 94–100. doi:10.1002/MRD.1080340115

www.publish.csiro.au/journals/rfd