UNIVERSITAT POLITÈCNICA DE VALÈNCIA

SISTEMA DE CULTIVO PARA MEJORAR LA VIABILIDAD DE EMBRIONES BOVINOS PRODUCIDOS *IN VITRO*

Tesis de doctorado (PhD)

Antonio Vinicio Murillo Ríos

Directores de Tesis:

Dr. Marta Muñoz Llamosas

Dr. Enrique Gómez Piñeiro

Valencia, Junio 2018

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Tesis presentada a la Universidad Politécnica de Valencia en cumplimiento parcial de los requisitos para el grado de Doctor en Filosofía (PhD)

Presentada por

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Firma

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Firma

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Valencia, Junio 2018

A Martina y Ramón

A mis padres, hermana y abuelos

« Ideas do not last long. We must do something with them »

«Las ideas no duran mucho. Hay que hacer algo con ellas»

Santiago Ramón y Cajal, 1852-1934

AGRADECIMIENTOS

A. Murillo-Ríos

Centro de Biotecnología Animal, Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Gijón, 33394

El presente trabajo de tesis doctoral no hubiese sido posible sin el apoyo y colaboración de todas las personas e instituciones que participaron oportuna y acertadamente en este proyecto. Por este motivo, a continuación quiero expresar mis más sinceros sentimientos de gratitud.

A mi director de tesis Dr. Enrique Gómez, por la oportunidad de formar parte de su equipo de trabajo. Bajo su dirección aprendí que el orden, la disciplina, la perseverancia y la rigurosidad son valores esenciales para desarrollar un trabajo de calidad. Gracias por todas las correcciones, las explicaciones, los consejos y la guía para culminar este trabajo.

A mi directora de tesis Dra. Marta Muñoz, por el tiempo, dedicación, ánimo y consejos de trabajo en el laboratorio y en la redacción científica. Me queda un gran ejemplo de lucha incansable por conseguir los objetivos que se plantean tanto en la vida académica como en la vida personal.

A Susana Carrocera Costa, por la dedicación, cordialidad y paciencia durante mi formación en el laboratorio de embriología, por el buen ambiente que brindas siempre, por las buenas influencias musicales y por todas las recomendaciones de la cultura asturiana.

A David Martín González por su trabajo técnico en los laboratorios, por dar guerra ante las adversidades del día a día en los trabajos experimentales y tener siempre un tiempo "pa echar unes rises". Gracias por todas las anécdotas de la "Llingua", de fútbol y de las tradiciones asturianas (de la Cuenca).

A la Dra. Marta Alonso y al Dr. Ángel Nistal del departamento de microscopía fotónica y proceso de imágenes de los Servicios Científico-Técnicos de la Universidad de Oviedo, por la valiosa colaboración técnica en los trabajos de microscopía confocal que componen esta tesis.

Al Dr. Félix Goyache y su grupo de trabajo (Isabel, Nuria é Iván) por todas las charlas de genética, estadística, antropología y política amenamente impartidas en estos cuatro años.

A mi tutor Dr. José Vicente Antón, por los ánimos para iniciar y cursar los estudios de doctorado en la UPV, sin tus palabras y guía durante los años de estudio previos quizá nunca me hubiera atrevido hacerlo. Gracias por la ayuda brindada con los trámites administrativos.

A Alberto Fernández Buznego y todo el equipo de operarios del Centro de Biotecnología Animal del SERIDA, por las amenas charlas, experiencias y bromas en medio del trabajo de campo.

Al servicio veterinario de la Cooperativa de Agricultores de Gijón, por la acertada colaboración en los trabajos de transferencia embrionaria.

A la Secretaría Nacional de Educación Superior, Ciencia y Tecnología (SENESCYT), por los recursos económicos predoctorales.

Al Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), por permitirme desarrollar mi trabajo de investigación como becario externo en la institución.

Al Ministerio de Economía, Industria y Competitividad del gobierno de España, por el financiamiento de los proyectos en los cuales se han realizado los trabajos de esta tesis. También por las ayudas de movilidad para participar en las reuniones del proyecto "Producción *in vitro* de embriones de animales de interés veterinario" convocatoria 2016.

A la dirección del "Máster universitario en biología y tecnología de la reproducción" de la Universidad de Oviedo, por permitirme formar parte en su plantilla docente.

To the European COST- Action 16119, *in vitro* 3-D total cell guidance and fitness (Cellfit) by the funds to participate in the training school and by the promotion of interdisciplinary and multicultural networking.

A los compañeros(as) de estudio en el centro de biotecnología animal del SERIDA: Eva Correia, Begoña Menéndez, Pilar Nieto, Alejandro Vázquez y Cristina Blanco, por el apoyo, los ánimos y las experiencias compartidas.

A Marcia Ferráz, Manuel Revilla, Meriem Hamdi, Beatriz Fernández, Ma. Jesús Sánchez, Celia de Frutos y Marta Vázquez por compartir sus experiencias estudiantiles y profesionales, por sus consejos y su sincera amistad.

A Niccolo, Paula, Antonio, Mateo, Mark, Ana y Eli, por el gran año de intercambio Erasmus, por compartir la cultura de sus países de origen y por los irrepetibles momentos compartidos en Gijón.

A Javier, Alejandra, Rafa, Álvaro y Nerea por haberme hecho huésped exclusivo en su Gijón del alma con extensión hasta las islas Canarias, su tiempo y amistad hicieron que me sienta como en casa desde el primer momento.

A Isra, Danny y Django por la afición musical y su talento en los ensayos, me ayudaron en gran medida a renovar energías, tan necesarias para sobrellevar este trabajo.

Finalmente a toda mi familia, nada hubiera sido posible sin su apoyo incondicional. Sus valores y enseñanzas han sido pilares fundamentales para alcanzar mis objetivos en la vida. A Martina por el soporte en la convivencia, por tu manera de ver la vida y por ser una estupenda amiga, compañera y esposa. Sin olvidarme de la gran alegría y aventura que iniciamos junto Ramón, va por ustedes.

RESUMEN

A. Murillo-Ríos

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En la última década, la producción de embriones bovinos *in vitro* se ha incrementado notablemente, convirtiéndose en la principal tecnología de embriones a escala mundial. En bovino, la producción de embriones *in vitro* incluye el diseño y preparación de medios de cultivo, los cuales son esenciales para dar soporte al desarrollo de ovocitos y embriones. Sin embargo, la producción de embriones *in vitro* aún continúa limitada por varios factores. El cultivo *in vitro* después de la fecundación es un período crítico para producir embriones de buena calidad y viabilidad. Además, el correcto desarrollo y mantenimiento de la gestación hasta el parto y la adecuada salud perinatal de los terneros están altamente correlacionados con los medios y sistemas de cultivo *in vitro*. En bovinos, el cultivo individual *in vitro* en condiciones definidas después de día-6 favorece el desarrollo embrionario y permite realizar diferentes análisis no invasivos del medio de cultivo. Por el contrario, los suplementos no definidos presentes en los medios de cultivo convencionales pueden reducir la repetibilidad de los análisis. Por lo tanto, el objetivo general de este trabajo de tesis doctoral fue optimizar un sistema de cultivo para mejorar la calidad y la viabilidad de embriones bovinos *in vitro*. Con este propósito se desarrollaron cuatro grupos de experimentos.

En primer lugar, evaluamos los efectos de la eliminación de proteína sobre el desarrollo de blastocitos durante un período corto de cultivo individual. La viabilidad del embrión a diferentes plazos fue analizada mediante supervivencia a la criopreservación y recuento diferencial de células embrionarias; porcentajes de gestación; y duración de la gestación, peso y morfometría de los terneros nacidos. Además, se realizó un análisis de expresión génica en blastocistos expandidos de Día-7 tanto antes como después de la criopreservación. De este

capítulo se puede concluir que el cultivo de embriones individuales durante 24 h en un medio libre de proteína produce menos blastocistos pero aumenta los porcentajes de nacimiento después de la vitrificación y la transferencia a receptoras.

A continuación se abordó la evaluación de la viabilidad de los blastocistos expandidos producidos en función de la cinética del embrión y la restricción de proteína durante un periodo corto en cultivo individual. Así, las mórulas y los blastocistos tempranos de día 6 se cultivaron individualmente con y sin proteína durante 24 h. El desarrollo y el contenido de lípidos se analizaron en blastocistos expandidos derivados de mórulas (M-XB) y de blastocistos tempranos (EB-XB). La expresión de genes implicados en el metabolismo lipídico, las respuestas al estrés y la apoptosis se analizaron en M-XB frescos y vitrificados, producidos con y sin proteína. Los índices de gestación, los porcentajes de nacimientos y el peso al nacimiento se registraron después de la transferencia de embriones. Los resultados indican que la cinética embrionaria y la vitrificación impactan en los fenotipos al nacimiento, al menos en el subconjunto de las terneras. Las alteraciones pueden involucrar la proteína exógena y la movilización de las reservas de lípidos.

Posteriormente, se investigó si una concentración muy baja de FCS (0.1%) en cultivo desde el día 1 hasta el día 6 podría mejorar los porcentajes de blastocisto temprano (EB) y, a continuación, aumentar los porcentajes de blastocisto expandido (XB) en día 7 después de un cultivo individual sin proteína. La calidad de los embriones producidos se evaluó en términos de supervivencia a la criopreservación, porcentaje de apoptosis, acumulación de lípidos y

transferencia a receptoras. Se concluye en este capítulo que la concentración mínima de FCS mejora los porcentajes de EB en el Día 6, permitiendo obtener más XB después de 24h de cultivo individual sin proteína. La calidad de los XB producidos con FCS es similar a los XB producidos con BSA en términos de apoptosis, acumulación de lípidos e índice de gestación.

Finalmente, el objetivo en el cuarto capítulo fue cuantificar la proteína total HDGF en el fluido uterino (FU) mediante *multiple reaction monitoring* (MRM), técnica que permite reconocer la proteína total. Además, analizamos los efectos de rHDGF en etapas embrionarias específicas con embriones bovinos de Día-6 cultivados *in vitro* con y sin proteína (BSA); y sobre la viabilidad de la preñez y los fenotipos de los terneros después de la transferencia de embriones a receptoras. Además, se cuantificó el ARNm de *HDGF* en células endometriales cocultivadas con un embrión macho o un embrión hembra. De este capítulo se puede concluir que el HDGF total cuantificado por MRM tendió a aumentar en el FU sin embriones, mientras que el sexo del embrión podría regular la expresión endometrial de HDGF. Sin embargo, se debe ser cauteloso con el uso de suplementos macromoleculares específicos en cultivo, ya que pueden contener el GF en estudio, como ocurre con la presencia de HDGF en la BSA comercial, lo que puede alterar los resultados de los experimentos. En última instancia, el uso de rHDGF es compatible con la gestación y el nacimiento de terneros normales.

ABSTRACT

A. Murillo-Ríos

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In the last decade, the *in vitro* production (*IVP*) of bovine embryos has been increased notably becoming the leading embryo technology worldwide. In cattle, *IVP* of embryos included the design and preparation of culture media, which are essential to support the development of oocytes and embryos. However, IVP of embryos still continues limited by several factors. *In vitro* culture after fertilization stage is a critical period to produce embryos of good quality and viability. Furthermore, a correct gestation development, it normal maintenance up to parturition and a suitable perinatal health of calves are highly correlated with media and *in vitro* culture systems. In bovine, individual *in vitro* embryo culture in chemically defined conditions after Day-6 benefits embryo development and different non-invasive analyses of culture medium can be realized. In contrast, undefined supplements in classical culture media can reduce analytical reliability. Then, the general aim of this thesis was to optimize an embryo culture system in order to improve quality and viability of *in vitro* bovine embryos. With this purpose, four groups of experiments were developed. First, we evaluated the effects of protein removal in a short period of individual culture on blastocyst development. The embryo viability was analyzed at different terms through survival to cryopreservation and differential cell counts; pregnancy rates and gestation length, weight and morphometry of calves born. In addition, gene expression analysis was performed on Day-7 expanded blastocysts both before and after cryopreservation. From this chapter it may be concluded that individual embryo culture for 24 h in a protein-free medium, reduced Day-7 expanded blastocyst rates but improved birth rates after vitrification and transfer to recipients.

Next, we evaluated the viability of produced expanded blastocyst in depends of embryo kinetics and protein restriction during a short step in individual culture. Thus, Day-6 morulae and early blastocysts were cultured individually with and without protein for 24 h. Development and lipid content were analysed in expanded blastocysts derived from morulae (M-XB) and from early blastocysts (EB-XB). Expression of genes involved in lipid metabolism, stress responses and apoptosis was analysed in fresh and vitrified/warmed M-XB produced with and without protein. Pregnancy rates, birth rates and birthweight were recorded after transfer of embryos. The results indicate that embryonic kinetics and vitrification impact birth phenotypes, at least in females. Alterations might involve exogenous protein and mobilisation of lipid stocks.

Later, we investigated whether a very low FCS concentration (0.1%) in culture from Day-1 to Day-6 would improve early blastocyst (EB) rates and, subsequently, increase expanded blastocyst (XB) rates on Day-7 after single culture in protein-free medium. The quality of embryos produced was evaluated in terms of survival to cryopreservation, apoptosis percentage, lipid accumulation and transfer to recipients. In conclusion, minute FCS concentration improves EB rates on Day-6 leading, after 24h of single culture without protein, to more XBs. The quality of XB produced with FCS compares well with XB produced with BSA in terms of apoptosis, lipid accumulation and pregnancy.

Finally, the aim of the last chapter was quantify total HDGF protein in uterine fluid (UF) by multiple reaction monitoring (MRM), and analysed effects of rHDGF on specific embryonic stages with Day-6 bovine embryos cultured *in vitro* with and without protein (BSA), and on pregnancy viability and calf

phenotypes after embryo transfer to recipients. In addition, mRNA abundance of HDGF in endometrial cells co-cultured with one male or one female embryo was quantified. From this chapter it may be concluded that total HDGF quantified by MRM tended to increase in UF without embryos, and the endometrial expression of HDGF can be regulated by the embryonic sex. Furthermore, rHDGF acts selectively on specific embryonic stages, but care should be taken with specific macromolecular supplements in culture, as the GF under study can be present, as it happens with presence of HDGF in commercial BSA, and the experiment results can be altered. Ultimately, the use of rHDGF is compatible with pregnancy and birth of normal calves.

RESUM

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En l'última dècada, la producció d'embrions bovins in vitro s'ha incrementat notablement, convertint-se en la principal tecnologia d'embrions a escala mundial. En boví, la producció d'embrions in vitro inclou el disseny i preparació de medis de cultiu, els quals són essencials per a donar suport al desenvolupament d'ovòcits i embrions. No obstant això, la producció d'embrions in vitro encara continua limitada per diversos factors. El cultiu in vitro, després de la fecundació, és un període crític per a produir embrions de bona qualitat i viabilitat. A més, el correcte desenvolupament i manteniment de la gestació fins al part i l'adequada salut perinatal dels vedells estan altament correlacionats amb els medis i sistemes de cultiu in vitro. En bovins, el cultiu individual in vitro en condicions definides després de día-6 afavoreix el desenvolupament embrionari i permet realitzar diferents anàlisis no invasius del medi de cultiu. Al contrari, els suplements no definits presents en els medis de cultiu convencionals poden reduir la repetibilitat dels anàlisis. Per tant, l'objectiu general d'este treball de tesi doctoral va ser optimitzar un sistema de cultiu per a millorar la qualitat i la viabilitat d'embrions bovins in vitro. Amb este propòsit es van plantejar quatre grups d'experiments.

En primer lloc, vam avaluar els efectes de l'eliminació de proteïna sobre el desenvolupament de blastocists durant un període curt de cultiu individual. La viabilitat de l'embrió a diferents terminis va ser analitzada per mitjà de la supervivència a la criopreservació i recompte diferencial de cèl·lules embrionàries; percentatges de gestació; i duració de la gestació, pes i morfometria dels vedells nascuts. A més, es va realitzar un anàlisi d'expressió gènica en blastocists expandits de dia-7 tant abans com després de la criopreservació. D'este capítol es pot concloure que el cultiu d'embrions

individuals durant 24 h en un medi lliure de proteïna produeix menys blastocists però augmenta els percentatges de naixement després de la vitrificació i la transferència a receptores.

A continuació es va abordar l'avaluació de la viabilitat dels blastocists expandits produïts en funció de la cinètica de l'embrió i la restricció de proteïna durant un període curt en cultiu individual. Així, les mòrules i els blastocists primerencs de dia 6 es van cultivar individualment amb i sense proteïna durant 24 h. El desenvolupament i el contingut de lípids es van analitzar en blastocists expandits derivats de mòrules (M- XB) i de blastocistos primerencs (EB-XB). L'expressió de gens implicats en el metabolisme lipídic, les respostes a l'estrès i l'apoptosi es van analitzar en M-XB frescs i vitrificats, produïts amb i sense proteïna. Els índexs de gestació, els percentatges de naixements i el pes al naixement es van registrar després de la transferència d'embrions. Els resultats indiquen que la cinètica embrionària i la vitrificació impacten en els fenotips al naixement, almenys en el subconjunt de les vedelles. Les alteracions poden involucrar la proteïna exògena i la mobilització de les reserves de lípids.

Posteriorment, es va investigar si una concentració molt baixa de FCS (0.1%) en cultiu des del dia 1 fins al dia 6 podria millorar els percentatges de blastocists primerenc (EB) i, a continuació, augmentar els percentatges de blastocists expandits (XB) en dia 7 després d'un cultiu individual sense proteïna. La qualitat dels embrions produïts es va avaluar en termes de supervivència a la criopreservació, percentatge d'apoptosi, acumulació de lípids i transferència a receptores. Es conclou en este capítol que la concentració mínima de FCS millora els percentatges d'EB en el Dia 6, permetent obtindre més XB després

de 24h de cultiu individual sense proteïna. La qualitat dels XB produïts amb FCS és semblant als XB produïts amb BSA en termes d'apoptosi, acumulació de lípids e índex de gestació.

Finalment, l'objectiu en el quart capítol va ser quantificar la proteïna total HDGF en el fluid uterí (FU) per mitjà de *multiple reaction monitoring* (MRM), tècnica que permet reconèixer la proteïna total. A més, analitzem els efectes de rHDGF en etapes embrionàries específiques amb embrions bovins de Dia-6 cultivats in vitro amb i sense proteïna (BSA); i sobre la viabilitat del prenyat i els fenotips dels vedells després de la transferència d'embrions a receptores. A més, es va quantificar l'ARNm de HDGF en cèl·lules endometrials cocultivades amb un embrió mascle o un embrió femella. D'este capítol es pot concloure que el HDGF total quantificat per MRM va tendir a augmentar en l'FU sense embrions, mentre que el sexe de l'embrió podria regular l'expressió endometrial de HDGF. No obstant això, s'ha de ser cautelós amb l'ús de suplements macromoleculars específics en cultiu, ja que poden contindre el GF en estudi, com ocorre amb la presència de HDGF en la BSA comercial, la qual cosa pot alterar els resultats dels experiments. En última instància, l'ús de rHDGF és compatible amb la gestació i el naixement de vedells normals.

ABREVIATURAS:

aaci	aminoácidos esenciales y no esenciales, citrato, mioinositol
ADN	ácido desoxirribonucleico
AGE	activación del genoma embrionario
ARNm	ácido ribonucleico mensajero
ATF4	activating transcription factor 4
ATP	adenosin trifosfato
BAX	bcl-2–associated X protein
Bb	bisbenzimida
BSP	proteínas del plasma seminal
BSA	albumina sérica bovina
°C	grados centígrados
cAMP	adenosín monofosfato cíclico
CIV	cultivo *in vitro*
COCs	complejos cumulo-ovocito
DAPI	4 ',6-diamino-2-fenilindol
DDIT3	DNA-damage-inducible transcript 3
EB	blastocisto temprano
FIV	fecundación *in vitro*
FCS	suero fetal bovino
FU	fluido uterino
G6PD	glucose-6-phosphate dehydrogenase
GFs	factores de crecimiento
g L^{-1}	gramos por litro
GPX1	glutathione peroxidase 1
HDGF	hepatoma derived growth factor
IA	inseminación artificial
IETS	international embryo transfer society
IFN-τ	interferón tau
IGF2R	insulin-like growth factor 2 receptor
IP	yoduro de propidio
M	mórula
MCI	masa celular interna
MIV	maduración *in vitro*
mOsM	miliosmomoles
MOET	multiple ovulation embryo transfer
mol L^{-1}	moles por litro
MRM	multiple reaction monitoring
NR	nile red
OPU	ovum pick-up
PKA	proteína quinasa A
PTK	tirosina quinasas
Ptyr–Ptase	tirosina fosfatasas
PVA	polivinil alcohol
sAC	adenil ciclasa soluble
SLC2A3	solute Carrier Family 2 member 3
SOD2	superoxide dismutase 2, Mitochondrial (formerly MnSOD)
SOF	medio sintético de fluido de oviducto
TD	transferencia directa
TE	tecnologías embrionarias
TEM	transición materno-embrionaria
TF	trofectodermo
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
µL	microlitro
v/c	vitrificación/calentamiento
XB	blastocisto expandido
ZP	zona pelúcida

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1 | INTRODUCCIÓN GENERAL

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1.1. Tecnologías embrionarias aplicadas en bovinos

El proceso de domesticación del ganado bovino (*Bos taurus* y *Bos indicus*) hace ≈11.000 años, provocó que la reproducción de estos animales pasase a estar controlada selectivamente por los seres humanos (Beja-Pereira *y cols.,* 2006). Más tarde, las tecnologías reproductivas desarrolladas en el ganado bovino permitieron perfeccionar el rendimiento de los sistemas de producción, mejorando sustancialmente su rentabilidad y su eficiencia.

En el ganado bovino, las tecnologías reproductivas embrionarias constituyen una de las herramientas más importantes para la investigación científica y la producción animal. Así, las tecnologías embrionarias (TE) han contribuido a mejorar notablemente la comprensión de los mecanismos fisiológicos básicos de la biología del desarrollo de los mamíferos, incluido el ser humano. Por otra parte, las TE aplicadas a la producción bovina, han permitido mejorar la capacidad reproductiva de la hembra y los esquemas de selección genética en las industrias láctea y cárnica.

La técnica de ovulación múltiple y transferencia de embriones (MOET, del inglés *Multiple Ovulation and Embryo Transfer*) y la técnica de producción *in vitro* de embriones son las TE más importantes dentro de los programas de reproducción bovina. El uso y la aplicación de las técnicas MOET y producción *in vitro* de embriones bovinos ha hecho posible desarrollar otras técnicas más complejas (por ejemplo clonación, transgénesis, producción de células madre) de gran interés en biomedicina (Bogliotti *y cols.,* 2018).

La técnica MOET consiste en la aplicación de un tratamiento hormonal para estimular los ovarios de una hembra donante para inducir una ovulación

múltiple. Una vez en celo, la donante es inseminada artificialmente y una semana más tarde se recogen los embriones producidos mediante lavado de los cuernos uterinos. Los embriones recogidos son después clasificados por su calidad y estadío para ser transferidos a receptoras sincronizadas o para criopreservarlos (Thibier, 2005).

Los programas MOET han hecho posible incrementar la intensidad de selección y reducir el intervalo generacional, acelerando la mejora genética en los esquemas de selección bovina. Sin embargo, desde su desarrollo original el rendimiento permanece prácticamente invariable, pues el número de embriones transferibles obtenidos por donante es reducido (promedio de 6.5; Perry*,* 2016). Otras desventajas incluyen la alta variabilidad de la respuesta a los tratamientos hormonales de ovulación múltiple (aproximadamente un 20% de las donantes no producen embriones), la necesidad de un amplio periodo de reposo entre cada tratamiento y la falta de eficiencia cuando se utiliza semen sexado (Hasler, 2003; Mikkola *y cols.,* 2017).

Desde la década de los 80 hasta 2015, los programas MOET representaron la TE más aplicada en el ganado bovino en un contexto global (Blondin, 2015). Por otra parte, el número de embriones producidos *in vitro* (PIV) se ha incrementado considerablemente desde los años 90 (Blondin, 2015), especialmente como resultado de las mejoras desarrolladas en el cultivo embrionario. Así, durante el año 2016 según los datos publicados por la sociedad internacional de transferencia de embriones (IETS), 668.837 embriones bovinos fueron PIV a escala mundial; superando por primera vez al número de embriones transferibles producidos *in vivo* mediante la técnica MOET (Perry, 2016) (Fig. 1.1).

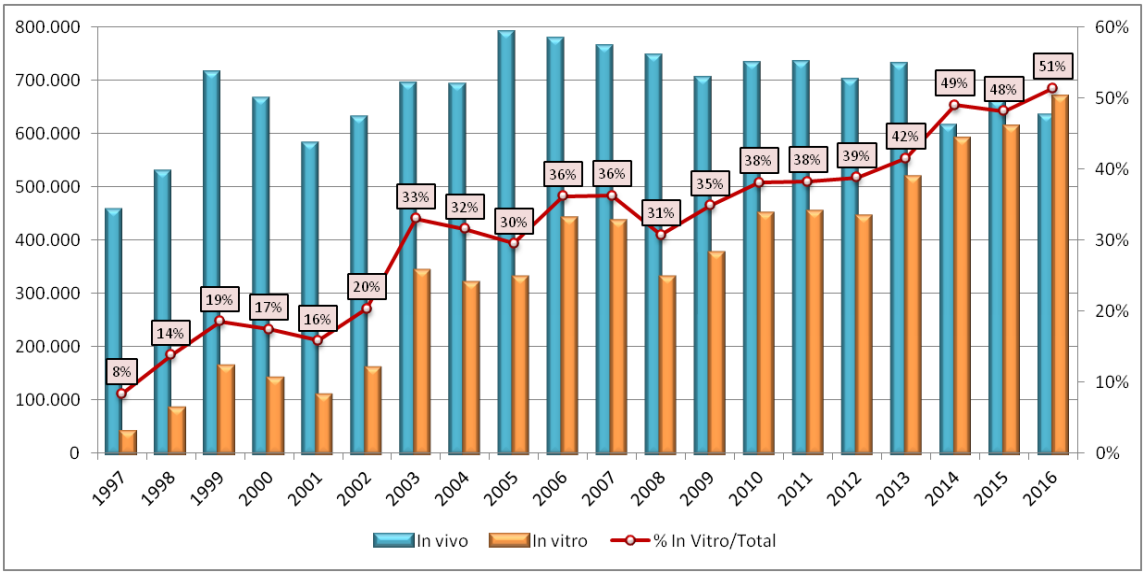

Fig. 1.1. Producción mundial de embriones bovinos *in vivo* (MOET) e *in vitro* entre el año 1997 y 2016. Adaptado de Blondin, 2015; y Perry, 2015, 2016.

Una de las estrategias para producir mejores embriones *in vitro* es tratar de imitar el ambiente y los eventos de maduración y fertilización ovocitaria, y desarrollo embrionario temprano, que ocurren en el trato reproductivo femenino. Los embriones bovinos *in vitro* pueden ser producidos a partir de ovocitos obtenidos de donadoras vivas, mediante la técnica de Ovum Pick-Up (OPU; Merton, 2003), o a partir de ovocitos de hembras sacrificadas mediante la punción de los ovarios obtenidos en el matadero (Galli *y cols.,* 2003).

Los protocolos de OPU para producir embriones bovinos *in vitro* pueden ser aplicados en una misma donadora una o incluso dos veces por semana haciendo que el número de crías que se puede obtener por unidad de tiempo sea superior a los conseguidos mediante la técnica MOET. Los embriones bovinos PIV se pueden obtener también mediante OPU de hembras gestantes (hasta el primer trimestre de gestación), animales tras dos o tres semanas de posparto, novillas pre-púberes o animales con ciertas patologías en el tracto

uterino (Baruselli, 2016). De igual manera, los ovocitos recuperados se pueden fecundar con espermatozoides de diferentes toros, lo que aumenta la variabilidad genética en la progenie y reduce los porcentajes de consanguinidad. Además, la producción *in vitro* de embriones bovinos permite un uso más eficiente del semen sexado, ya que con una sola pajuela de semen se puede fecundar un amplio número de ovocitos (Pontes *y cols.,* 2010; Morotti *y cols.,* 2014).

A pesar de las ventajas y el potencial de la producción *in vitro* de embriones bovinos, aun se busca mejorar los porcentajes de producción de embriones viables. Cuando se usan ovocitos madurados *in vivo* se pueden obtener porcentajes de blastocistos de hasta el 80% (Blondin *y cols.,* 2002). No obstante, cuando los ovocitos son madurados *in vitro* sólo entre el 30-40% de los ovocitos tratados alcanzan el estadio de blastocisto (Lonergan y Fair, 2014).

Los embriones PIV son más sensibles a la criopreservación que los embriones desarrollados en el animal vivo (Rizos *y cols.,* 2008; Sudano *y cols.,* 2011). Los patrones de expresión génica también se encuentran alterados en los embriones PIV en comparación con los embriones producidos *in vivo* (Lonergan, 2003; Gad, 2012). Además, se han observado cambios en el transcriptoma de embriones bovinos *in vitro* producidos en diferentes condiciones de cultivo (Nieman, 2000; Cagnone *y cols.,* 2012; van der Weijden *y cols.,* 2017).

De igual manera, los índices de gestación que se obtienen con embriones PIV transferidos a receptoras son más bajos que los obtenidos con embriones producidos *in vivo* (Pontes *y cols.,* 2009; Siqueira *y cols.,* 2009). Además, los

embriones bovinos PIV ocasionan elevados porcentajes de abortos tempranos (Stewart *y cols.,* 2011), anormalidades fetales (Young, 1998; Farin *y cols.,* 2006), distocia y mortalidad perinatal (van Wagtendonk-de Leeuw *y cols.,* 1998; Farin, 2010; Bonilla *y cols.,* 2014).

En vacas de leche, la calidad del folículo preovulatorio ha sido identificada como una de las principales causas de las pérdidas embrionarias al inicio de la gestación (Lonergan *y cols.,* 2016; Wiltbank *y cols.,* 2016). En este sentido, el proceso de maduración *in vitro* (MIV) determina en gran medida el éxito de la fertilización y el posterior desarrollo embrionario (Nuttinck *y cols.,* 2017; Botigelli *y cols.,* 2017).

Varios estudios realizados en mamiferos han demostrado que la calidad de los embriones bovinos PIV está asociada a las condiciones del cultivo, durante los estadíos de zigoto y blastocisto (Rizos *y cols.,* 2002a; Fleming *y cols.,* 2015; Hansen *y cols.,* 2015). De esta manera, el cultivo, que sigue a la fecundación, también es un periodo crítico en el proceso de producción de embriones bovinos *in vitro*, e incide directamente en la calidad del embrión y también en la salud del feto y del ternero nacido (Fleming *y cols.,* 2015; Duranthon *y cols.,* 2018). La calidad de los embriones PIV todavía es hoy deficiente en comparación con sus homólogos *in vivo*. Por lo tanto, es esencial optimizar los sistemas de PIV de embriones bovinos no solo para producir mayores porcentajes de blastocitos, sino, lo que es más importante, blastocistos de mejor calidad capaces de producir gestaciones a término con una descendencia saludable.

1.2. Producción de embriones bovinos *in vitro*

Generalmente en bovino, la producción de embriones *in vitro* implica tres pasos secuenciales: la MIV de ovocitos, la fecundación *in vitro* (FIV) de los ovocitos madurados y el cultivo *in vitro* (CIV) de los ovocitos fecundados hasta el estadio de blastocisto. En cada paso de la producción de embriones *in vitro*, las condiciones físico-químicas de los medios MIV, FIV y CIV deben optimizarse para permitir la maduración, capacitación e interacción de gametos, así como el desarrollo de embriones.

1.2.1. Maduración *in vitro*

Los ovocitos inmaduros se pueden obtener de vacas y novillas vivas mediante OPU ó a partir de ovarios recogidos en matadero. Los folículos antrales (3-8 mm de diámetro) son aspirados y los complejos cumulo-ovocito (COCs, del inglés *cumulus-oocyte complex*) que se obtienen se seleccionan en la práctica por su morfología. Así, únicamente los ovocitos que están rodeados de una a tres o más capas de células del cumulus y que presentan un citoplasma homogéneo se escogen para MIV (Fig. 1.2).

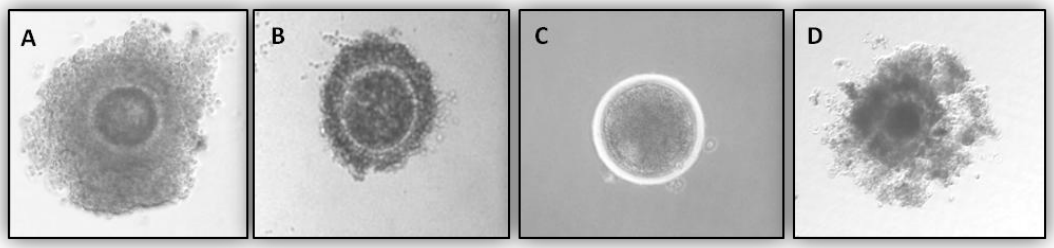

Fig. 1.2. Clasificación de ovocitos bovinos recuperados de ovarios antes del proceso de maduración *in vitro*. A: ovocito con más de tres capas de células del cumulus, B: ovocito con una a tres capas de células del cumulus, C: ovocito sin células del cumulus, D: ovocito con células del cumulus expandidas.

Las células del cumulus y el ovocito mantienen un intercambio metabólico intenso mediado por factores paracrinos y a través de uniones gap. En los COCs aislados de folículos antrales tempranos, la comunicación por medio de uniones gap funcionales promueve el crecimiento del ovocito, el silenciamiento gradual del transcriptoma, la remodelación de la cromatina y la adquisición de competencia; todas controladas mediante el adenosín monofosfato cíclico (cAMP, del inglés *cyclic adenosine monophosphate*) del ovocito (Luciano *y cols.,* 2011, 2018).

La maduración nuclear y citoplasmática de los ovocitos es determinante para la fecundación y el desarrollo embrionario posterior. La maduración nuclear implica la reanudación de la división meiótica hasta metafase II y la extrusión del primer corpúsculo polar, mientras que la maduración citoplásmica incluye la redistribución de orgánulos (Ferreira *y cols.,* 2009) y la acumulación de ARNm, proteínas, sustratos y nutrientes (Watson, 2007).

Se ha demostrado que factores como el origen de los ovocitos (OPU o matadero) y el tamaño de los folículos, así como la edad, la raza y la salud de las donantes influyen en la competencia *in vitro* de los ovocitos bovinos (Gilchrist *y cols.,* 2004; Luciano y Sirard, 2018). La competencia en el desarrollo de los ovocitos, a corto plazo, se define generalmente como la capacidad del gameto femenino para madurar, ser fertilizado y mantener el desarrollo del embrión hasta el estadío de blastocisto (Conti y Franciosi, 2018) y durante la elongación (Nuttinck *y cols.,* 2017). Sin embargo, a largo plazo, la competencia de los ovocitos se relaciona con la capacidad de producir gestaciones a término y generar una descendencia sana (Sirard *y cols.,* 2006; Lonergan *y cols.,* 2016; Nuttinck *y cols.,* 2017). La reducción en los porcentajes

de producción de blastocistos se puede atribuir en parte a la heterogeneidad de la población de ovocitos que normalmente se recupera para maduración *in vitro*, ya que frecuentemente no provienen de folículos preovulatorios (Wrenzycki, 2016). Los ovocitos completan la fase de MIV al cabo de 22 a 24 h; entonces se puede iniciar el proceso de fecundación. En bovinos, aproximadamente el 90% de los ovocitos inmaduros de buena calidad maduran *in vitro* con éxito (Mermillod *y cols.,* 2006).

1.2.2. Fecundación *in vitro*

La FIV implica la unión de los gametos femeninos y masculinos durante un período de coincubación. El semen bovino congelado tiene que ser descongelado y tratado adecuadamente antes de la FIV. Los métodos de separación se usan para eliminar el diluyente espermático y los espermatozoides muertos, consiguiendo así espermatozoides vivos y móviles. Comúnmente, se usan dos métodos para la separación de espermatozoides: el método "swim-up" basado en las características de motilidad espermática (Parrish *y cols.,* 1986) y el método de gradiente discontinuo basado en las diferentes densidades de los espermatozoides vivos y muertos (Saeki *y cols.,* 1991).

Los medios y protocolos comúnmente utilizados para la FIV en bovino descritos por Parrish *y cols.,* (1986) no han sufrido en general modificaciones considerables. Los espermatozoides necesitan ser capacitados para fertilizar ovocitos durante la FIV. La capacitación de los espermatozoides supone la modificación de la membrana espermática y cambios en la motilidad del

gameto. La capacitación espermática se induce con la suplementación de agentes de capacitación a los medios de fecundación dependiendo de las características espermáticas de cada toro. Uno de los agentes de capacitación usado comúnmente es la heparina. La heparina se une a las proteínas del plasma seminal (BSP, del inglés *bovine seminal plasma proteins*) y las elimina de la membrana plasmática junto con el colesterol y los fosfolípidos asociados. La heparina reduce la capacidad de los espermatozoides para expulsar Ca^{2+} a través de la calcio-ATPasa. También se produce una absorción de Ca^{2+} extracelular en el acrosoma, lo que da lugar a un incremento del calcio intracelular, a la vez que se induce la salida de H^+ y entrada de HCO_3^-. Los cambios de HCO_3^- y H^+ aumentan el pH intracelular (pH_i) y tanto el HCO_3^- como el incremento del pH_i estimulan la adenil ciclasa soluble (sAC, del inglés *soluble adenylate cyclase*) de los espermatozoides. El cAMP resultante activa la proteína quinasa A (PKA, del inglés *protein kinase A*), la cual mediante comunicación cruzada estimula a su vez las tirosina quinasas (PTK, del inglés *protein tyrosine kinases*) e inhibe las tirosina fosfatasas (Ptyr–Ptase, del inglés *protein tyrosine phosphatases*) produciéndose finalmente, la transferencia de un grupo fosfato desde el ATP a los residuos de tirosina presentes en diversas proteínas. El incremento de calcio intracelular estimula aún más la sAC y la inhibición de Ptyr–Ptase (Fig. 1.3) (Breininger *y cols.,* 2010; Parrish, 2014). Recientemente, se ha descrito un nuevo fenómeno biológico asociado a la capacitación de espermatozoides mamíferos *in vitro*, el cual implica una redistribución de iones de zinc en el espermatozoide (Kerns *y cols*., 2018).

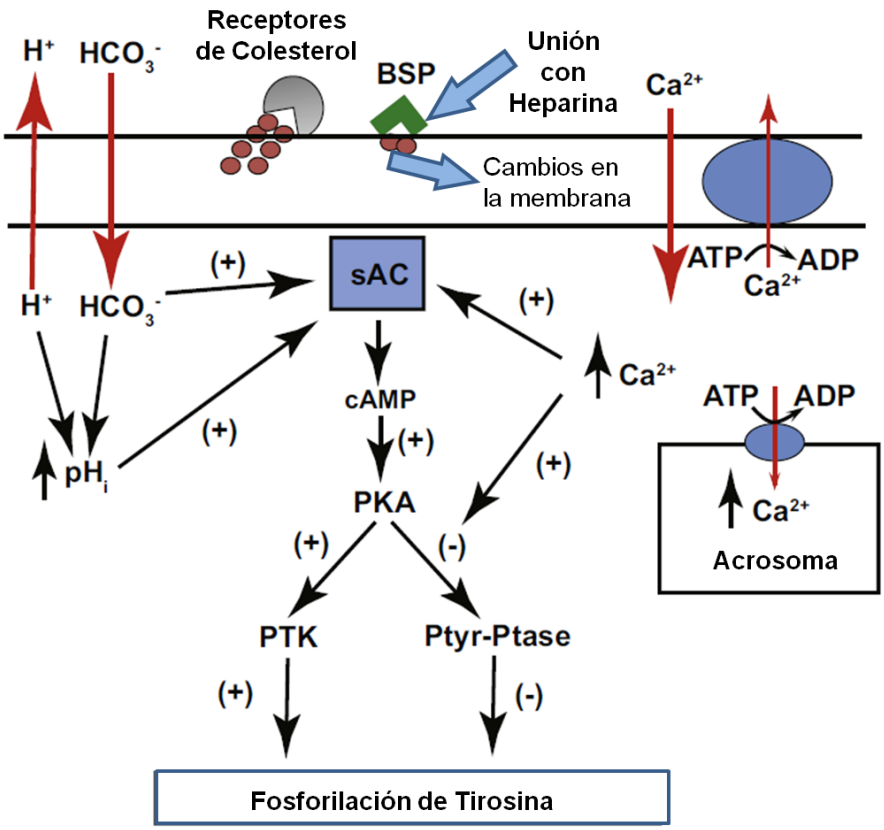

Fig. 1.3. Esquema de los procesos intracelulares inducidos por la heparina durante la capacitación de espermatozoides bovinos. (Adaptado de Parrish, 2014).

Otros agentes, como la penicilamina, hipotaurina y epinefrina (PHE), se usan para mejorar los porcentajes de penetración espermática cuando los espermatozoides son de baja calidad (Susko y Parrish, 1990; Parrish, 2014).

El uso de semen sexado posibilita la producción de embriones *in vitro* del sexo deseado, de gran interés en las explotaciones bovinas de leche (hembras de reemplazo) y carne (machos para engorde). El semen bovino puede ser sexado a partir de eyaculados frescos (sexaje convencional) y también de dosis previamente congeladas (sexado inverso de semen; Underwood, 2010). El método de separación espermática se basa en las diferencias del contenido de ADN entre el cromosoma X y el cromosoma Y, medido por citometría de flujo. Sin embargo, se han descrito porcentajes de blastocistos *in vitro* más bajas con

el uso de semen sexado frente a las obtenidas con semen convencional, aunque con un marcado efecto individual de cada toro (Trigal *y cols.,* 2012).

En bovinos, la concentración final de espermatozoides utilizada en la FIV varía entre 0.5 y $2x10^6$ espermatozoides por mililitro de medio de fecundación. Esta concentración debe optimizarse para cada toro y a veces para cada eyaculado de un mismo toro. Comúnmente, los ovocitos maduros se incuban con espermatozoides móviles capacitados durante 18-20 h, aunque una coincubación de 6h basta para una fecundación efectiva (Galli, 2003; Gordon *y cols.,* 2003).

En la FIV, los espermatozoides deben atravesar las células del cumulus que rodean al ovocito hasta llegar a la zona pelúcida (ZP). La zona pelúcida es una matriz extracelular gruesa y translúcida que rodea al ovocito y al embrión hasta el momento de la eclosión. En bovinos, la ZP está compuesta por tres glicoproteínas llamadas ZPA/ZP2, ZPB/ZP4, ZPC/ZP3 (Goudet *y cols.,* 2008; Avilés *y cols.,* 2010; Suzuki *y cols.,* 2015). El contacto entre el espermatozoide y la ZP induce la reacción acrosómica (exocitosis) del espermatozoide, el cual terminará fusionándose con el ooplasma y activando el ovocito dando inicio a la formación de los pronúcleos (Sutovsky., 2011). Al tiempo, se activa el bloqueo de la polispermia mediante la liberación de moléculas contenidas en los gránulos corticales que producen importantes modificaciones químicas, particularmente en la zona pelúcida, el espacio perivitelino y el oolema del ovocito. Recientemente, la exocitosis de gran cantidad de iones de zinc (*zinc spark*) emitidos por el ovocito e inducido por los espermatozoides durante la fertilización ha sido propuesto como un nuevo biomarcador de la calidad del embrión y del potencial de desarrollo embrionario en ratones (Que y cols.,

2015; Zhang *y cols.,* 2016). Los porcentajes de fecundación con semen convencional pueden alcanzar promedios de un 80% o más, mientras que con semen sexado se han reportado porcentajes de fecundación de hasta 60% (Yang Li *y cols.,* 2018).

1.2.3. Cultivo *in vitro*

El CIV de embriones bovinos comprende el desarrollo desde el estadío de zigoto hasta los diferentes estadíos de blastocisto. En su fase inicial, el desarrollo embrionario temprano está regulado por transcritos de ARN maternos y por proteínas producidas y almacenadas durante la ovogénesis (Graf *y cols.,* 2014). Las divisiones mitóticas transforman el zigoto en un embrión multicelular a través de un proceso llamado segmentación. Cada célula generada se denomina blastómero y su tamaño disminuye conforme avanza la división celular, sin afectar el tamaño del embrión hasta el estadío de blastocisto.

En los mamíferos, la división celular después del estadío de dos células suele ser asincrónica, generando embriones con un número irregular de blastómeros (Prados, 2012). Al comienzo del desarrollo embrionario los blastómeros son totipotentes, lo que significa que pueden ser inducidos a convertirse en cualquier tipo de célula fetal o adulta. Las proteínas y los transcritos maternos se degradan gradualmente mientras los transcritos del embrión comienzan a sintetizarse una vez que se produce la transición materno-embrionaria (TEM). La TEM incluye la activación del genoma embrionario (AGE; Graf *y cols.,* 2014). En bovino, la AGE se produce entre los estadios de 8 a 16 células

(Cagnone y Sirard, 2013), aunque la heterogeneidad del transcriptoma en células individuales de embriones bovinos de día dos y tres de cultivo después de la fecundación, sugiere un desarrollo asincrónico de los blastómeros durante la fase de mayor AGE (Lavagi *y cols.,* 2018).

Con el avance de las divisiones celulares, los blastómeros periféricos se aplanan y se inicia una síntesis de proteínas específicas que favorecen uniones intercelulares más estrechas, lo cual genera un mayor contacto entre las células embrionarias. El embrión pasa a llamarse mórula y la compactación celular se hace más evidente a medida que avanza el desarrollo embrionario. La transcripción del genoma embrionario después del TEM es extremadamente dinámica y controla la compactación de la mórula y el posterior desarrollo del blastocisto (Rodríguez-Zas *y cols.,* 2008).

El estadío de mórula se caracteriza por la formación de uniones membranosas estrechas y oclusivas (uniones tight) (Gualtieri *y cols.,* 1992; Boni *y cols.,* 1999) entre los blastómeros periféricos, mientras que entre los blastómeros internos se forman estrechas uniones de membrana que propician la comunicación intercelular (uniones gap) (Wrenzycki *y cols.,* 1996; Boni *y cols.,* 1999). La adquisición de uniones gap durante la compactación proporciona una mejor coordinación entre las células embrionarias en términos de metabolismo, transmisión de señal y respuesta a las condiciones externas (Cagnone y Sirard, 2016). Una vez que el embrión está completamente compactado se forma gradualmente una cavidad llena de líquido (blastocele) que aumenta conforme avanza el desarrollo embrionario. En esta fase el embrión pasa a denominarse blastocisto.

El blastocisto está formado por células que forman una capa externa o trofectodermo (TF), una masa celular interna (MCI) y el blastocele. El blastocele se forma progresivamente en respuesta a la alta concentración de cationes generada por la actividad de la bomba Na+/K+ en las células del TF. Las células del TF formarán la parte fetal de la placenta y las membranas embrionarias adicionales. Las células de la MCI son pluripotentes y producirán todos los tejidos embrionarios y una parte de las membranas extra embrionarias (Wang y Dey, 2006). A medida que el blastocele se expande, la zona pelúcida se hace cada vez más fina y el tamaño del embrión se incrementa (Fig. 1.4).

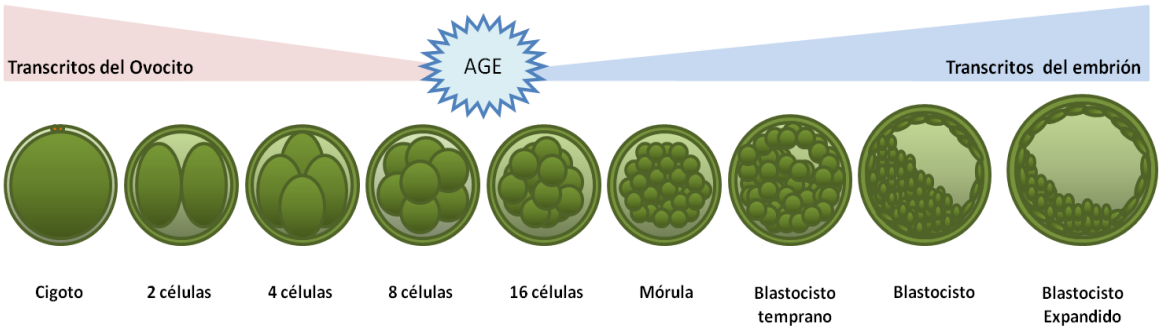

Fig. 1.4. Desarrollo embrionario bovino. AGE: activación del genoma embrionario.

En la producción de embriones bovinos *in vitro*, el blastocisto expandido (XB) es el estadío más resistente a la criopreservación, produciendo mejores índices de gestación en comparación con otros estadíos embrionarios anteriores o posteriores (Kubisch *y cols.,* 2004; Randi *y cols.,* 2016). Tras una notable expansión, el embrión *in vitro* se libera de la adelgazada zona pelúcida mediante ciclos alternativos de contracción y expansión, en un proceso que se denomina eclosión. No está claro si en el desarrollo *in vitro* proteasas del TF pueden contribuir a degradar la ZP, tal como parece ocurrir en el útero *in vivo.*

1.2.4. Características de los medios de cultivo *in vitro* para bovinos

Se han diseñado diferentes sistemas de CIV con el objetivo de imitar en la medida de lo posible las condiciones fisiológicas *in vivo* de los embriones bovinos. Por lo general, los medios de cultivo utilizados para PIV de embriones bovinos son una mezcla de componentes que pueden incluir: agua, sales, fuentes de energía, macromoléculas, aminoácidos, sustancias tampón y antioxidantes.

La osmolaridad y pH del medio CIV son factores importantes para el desarrollo de embriones de mamíferos. En el tracto reproductivo de la vaca, el fluido oviductal y uterino presentan osmolaridades alrededor de 290 y 315 mOsM respectivamente (Menezo y Guerin, 1997; Baltz, 2001) y se ha señalado que los embriones bovinos soportan osmolaridades de entre 250 y 300 mOsM en los medios de CIV (Baltz, 2001; Gasperin *y cols.,* 2016). El fluido oviductal y uterino muestran valores de pH de 7.6 y 6.9 respectivamente (Hugentobler *y cols.,* 2004), mientras que la mayoría de los medios para producción de embriones bovinos *in vitro* tienen un pH de 7.3 a 7.4, excepto en los medios de fecundación, que generalmente oscila entre 7.5 y 7.6 (Hasler y Barfield, 2014).

Diferentes tipos de aceite mineral o siliconas son un componente importante en varios sistemas de cultivo de gametos y embriones de mamíferos. Por lo general, se puede usar aceite mineral para recubrir los medios y evitar la evaporación, estabilizar el pH y la temperatura y evitar cambios de osmolaridad. Los aceites para la producción de embriones *in vitro* están constituidos a base de silicio o carbono (ambos de origen mineral) y pueden volverse tóxicos para los embriones en cualquier momento (Martínez *y cols.,*

2017). La exposición a la luz durante la producción de embriones bovinos *in vitro* debe limitarse a mínimos (Vatja, 2010).

El medio base más utilizado en el CIV de embriones bovinos es el medio sintético de fluido de oviducto (SOF) descrito por Tervit *y cols.,* (1972). Frecuentemente, el medio SOF se ha suplementado con aminoácidos esenciales y no esenciales, citrato, mioinositol (SOFaaci, Holm *y cols.,* 1999) y fuentes de proteína como suero o BSA. El suero tiene un efecto bifásico sobre el desarrollo embrionario, inhibiendo los primeros estadios y estimulando el desarrollo de mórulas y blastocistos (Pinyopummintr y Bavister 1994, Thompson y col 1998, Gómez y Diez 2000, Holm y col 2002). Sin embargo, se ha demostrado que el suero puede ser perjudicial para el desarrollo *in vitro* de embriones, ya que disminuye drásticamente la calidad embrionaria (Heras *y cols.,* 2016). Así, la presencia de suero en el cultivo embrionario modifica los orgánulos celulares, degrada las mitocondrias, altera la expresión génica, aumenta el contenido de lípidos y reduce la supervivencia a la criopreservación (Abe *y cols.,* 2002; Rizos *y cols.,* 2003; Gómez *y cols.,* 2008; Sudano *y cols.,* 2011). Además, una vez que los embriones son transferidos a receptoras los riesgos de alteraciones fetales, distocias y problemas perinatales se incrementan con el uso de suero durante el CIV (Farin *y cols.,* 2001; Lazzari *y cols.,* 2002).

La BSA es la proteína más abundante en el tracto reproductivo femenino y ha sido usada frecuentemente en los CIV como reemplazante del suero (Leese, 1988). No obstante, la composición de los tipos y lotes de BSA no es homogénea. La BSA es un compuesto de proteína no definido ya que contiene lípidos, aminoácidos, hormonas, péptidos, metales y otras moléculas no

conocidas de bajo peso molecular que determinan una variación significativa entre lotes (O'Neill, 2008; Francis, 2010). Estas variaciones afectan directamente al desarrollo y calidad de los embriones *in vitro* (Rorie *y cols.,* 1994; Francis, 2010). La BSA actúa limitando los efectos de toxinas y regulando el potencial óxido-reductor, el pH y la osmolaridad en los medios de cultivo (Gardner, 2008). No obstante, El uso de medios químicamente definidos tiene ventajas, como son la mayor repetibilidad de los ensayos (entre y dentro de laboratorios) y la no incorporación de moléculas con actividad biológica desconocida que pueden alterar los resultados de diferentes análisis de los medios de cultivo (Summers y Biggers, 2003; Gómez *y cols.,* 2016).

Uno de los reemplazantes macromoleculares sintéticos más utilizados en los medios de CIV de embriones mamíferos es el alcohol de polivinilo (PVA; Bavister, 1981). El PVA se ha usado como agente surfactante en medios de lavado y mantenimiento de embriones que están formulados para no incluir componentes de origen animal. Varios estudios realizados en bovino han utilizado el PVA como única macromolécula durante el CIV (Duque *y cols.,* 2003; Orsi y Leese, 2004) y se han obtenido preñeces y terneros saludables (Holm *y cols.,* 1999; Hidalgo *y cols.,* 2005; Lim *y cols.,* 2007). No obstante, la falta de proteína durante todo el periodo de CIV es perjudicial para el desarrollo embrionario ya que se obtienen menores porcentajes de blastocistos (Krisher y Bavister, 1999; Orsi y Leese, 2004).

Los embriones de mamíferos secretan factores tróficos al medio de cultivo, por lo que tienden a crecer mejor cuando se cultivan en grupo que individualmente (Gardner *y cols.,* 1994). La capacidad para el desarrollo embrionario, la calidad del embrión y la sensibilidad a la criopreservación también se ven afectadas

por el cultivo individual prolongado, y pocos autores describen porcentajes de desarrollo hasta blastocisto similares o superiores a los cultivos en grupo (Goovaerts *y cols.,* 2010). En los programas de OPU para producir embriones bovinos *in vitro* frecuentemente se requieren sistemas de cultivo para grupos reducidos de embriones. Además, el cultivo individual de embriones es imprescindible para realizar análisis moleculares no invasivos (Bunel *y cols.,* 2015; Gómez *y cols.,* 2016; Parker *y cols.,* 2017). Por lo tanto, es común ajustar el volumen de medio al número de embriones mediante el cultivo en microgotas. El volumen de las microgotas suele variar entre 10 a 50 µL y por lo general un volumen menor a 10 µL por embrión compromete seriamente el desarrollo de blastocistos en cultivos individuales completos sin renovación de medio (Lane y Gardner, 1992; Carolan *y cols.,* 1996). Aunque los embriones de mamíferos pueden ser cultivados individualmente en microgotas de 2 µL es necesaria la renovación del medio de cultivo cada 48h (Kelley y Gardner, 2017).

Para superar los efectos negativos de un cultivo individual completo, se ha descrito un cultivo individual más corto, de tan solo 24 h a partir de embriones seleccionados en día 6 (Muñoz *y cols.,* 2014a). De esta manera, el cultivo individual no afecta a la producción de blastocistos comparado con un cultivo realizado enteramente en grupo y además facilita la aplicación de análisis no invasivos en los medios de cultivo, los cuales pueden usarse para predecir índices de gestación (Muñoz *y cols.,* 2014a, 2014b) y diagnosticar el sexo del embrión (Muñoz *y cols.,* 2014c).

1.2.5. Factores de crecimiento para el cultivo *in vitro* de embriones bovinos

En el ambiente materno en que se desarrolla el embrión bovino *in vivo* figuran diversos factores de crecimiento (GFs, del inglés *Growth factors*), citoquinas, hormonas y otras moléculas reguladoras (Spencer y Bazer, 2004; Muñoz *y cols.,* 2012) que tienen funciones determinantes en el desarrollo embrionario y que pueden variar en función del sexo del embrión (Gómez *y cols.,* 2013). Los GFs pueden ser producidos por el embrión y/o el tracto reproductivo e influyen en el desarrollo embrionario de una manera autocrina o paracrina. La suplementación del CIV con sustancias sintéticas como GFs maternos recombinantes es una alternativa para la optimización de medios de cultivo especialmente cuando se pretende cultivar embriones en condiciones definidas.

En bovino, estudios previos han identificado un gran número de GFs producidos por el oviducto y el útero (Block *y cols.,* 2011; Muñoz *y cols.,* 2017). Además, la suplementación del medio CIV con GFs producidas por el endometrio de la vaca mejora el desarrollo del embrión *in vitro* y la supervivencia embrionaria después de la transferencia a receptoras (Larson *y cols.,* 1992; Sirisathien *y cols.,* 2003; Neira *y cols.,* 2010; Block *y cols.,* 2011; Fields y cols., 2011; Sakagami *y cols.,* 2012; Gómez *y cols.,* 2014; Moreno *y cols.*, 2015; Muñoz *y cols.,* 2017).

Es importante tener en cuenta que la acción de los GFs en el CIV puede ser dependiente del estadío del embrión, por ejemplo, la suplementación de activina A en el CIV de embriones bovinos mejora el desarrollo de blastocistos solo a partir del estadío de mórula en día 5, y no antes. Sin embargo, el número

de células del TE disminuye con la activina A tanto antes como después del estadío de mórula de día 5, lo que sugiere que la activina A inhibe la diferenciación de TE (Trigal *y cols.,* 2011).

De igual manera, Gómez *y cols.,* (2014) demostraron que el HDGF (del inglés, hepatoma-derived growth factor) está presente en el fluido uterino de vacas en los primeros días de gestación y es sintetizado tanto por el endometrio como por el embrión, pudiendo actuar de forma autocrina y/o paracrina promoviendo el desarrollo temprano de embriones *in vitro*. Los efectos embriotróficos de los GFs pueden variar dependiendo del estadío embrionario. Así, la presencia de HDGF recombinante (rHDGF) disminuyó el desarrollo embrionario in vitro cuando se agregó en el Día 5 de cultivo, pero aumentó el desarrollo cuando se agregó en el Día 6 (Gómez *y cols.,* 2014).

1.2.6. Cocultivo de células endometriales y embriones

El endometrio bovino constituye la capa más interna de la mucosa del útero. Está compuesto por áreas carunculares e intercarunculares que a su vez están formadas mayoritariamente por tres tipos de células: 1) células luminales epiteliales, 2) células glandulares epiteliales (ausentes en el área caruncular) y 3) células del estroma (Gray *y cols.,* 2001), además de vasos sanguíneos y linfocitos. En la vaca, el endometrio sufre cambios estructurales durante el ciclo estral en respuesta a las hormonas circulantes y es responsable de la secreción de numerosas citoquinas, factores de crecimiento y proteínas, las cuales son secretadas desde el epitelio glandular hacia la luz uterina (Ulbrich *y cols.,* 2013). Por lo tanto, es necesario contar con un mejor conocimiento del

funcionamiento del tracto genital para mejorar las condiciones del cultivo de embriones *in vitro*.

El cultivo *in vitro* de células endometriales se ha desarrollado tratando de mantener características estructurales y morfológicas típicas del órgano *in vivo* (Ulbrich *y cols.,* 2011). Además, el desarrollo de técnicas de cocultivo de células endometriales bovinas y embriones *in vitro* ha permitido abordar el estudio de las interacciones materno-embrionarias durante las primeras etapas del desarrollo embrionario, estudio cuya realización no es posible o es difícil en el animal vivo. Así, el reducido tamaño del embrión durante las primeras etapas del desarrollo en el útero (mórula-blastocisto) hace que el endometrio adyacente al embrión sea difícil de identificar y aislar, complicando el estudio del microambiente uterino que envuelve al embrión *in vivo* (Hunter 1994). Los inconvenientes pueden ser más pronunciados con los movimientos del embrión (Muñoz *y cols.,* 2012) y la alta densidad en el fluido uterino (Gómez *y cols.,* 2013).

En bovino, se ha propuesto la transferencia múltiple de embriones (Gómez y Muñoz, 2015) como alternativa capaz de detectar moléculas y efectos en el fluido uterino que no serían cuantificables con la presencia de un solo embrión. Por otro lado, Binelli *y cols.,* (2017) evaluaron el transcriptoma en diferentes secciones y cortes del endometrio bovino en presencia de un embrión de día 7 producido *in vivo* y encontraron que el patrón de expresión de genes específicos en el endometrio bovino responde a una programación tanto dependiente como independiente del embrión. La programación del endometrio dependiente del embrión requiere proximidad física endometrio-embrión. Por lo

tanto, el espacio donde tiene lugar la interacción endometrio-embrión es reducido y difícil de aislar para su estudio.

En este sentido, es importante destacar que el cultivo de células endometriales *in vitro* es un modelo alternativo que contribuye al estudio fisiológico o patológico del endometrio bovino (Asselin *y cols.,* 1996; Cronin *y cols.,* 2012) y que además favorece el análisis de las interacciones materno-embrionarias realizado en presencia de un solo embrión (Gómez *y cols.,* 2018).

1.3. Criopreservación de embriones producidos *in vitro*

La criopreservación de embriones bovinos producidos *in vitro* se realiza rutinariamente tanto en laboratorios de investigación como en laboratorios comerciales. La criopreservación es un proceso que consiste en someter a los embriones a temperaturas muy bajas, lo que ayuda a reducir la actividad fisiológica de cada célula embrionaria para conservarlos vivos durante largos periodos de tiempo.

En bovinos, la criopreservación de embriones contribuye a reducir los costes en las ganaderías, evita la dependencia de la actividad reproductiva cíclica y del estado fisiológico de los animales y es una necesidad cuando existen excedentes de embriones o escasez de receptoras. De igual manera, la criopreservación facilita la gestión y el uso de embriones para limitar la deriva genética (cambio en las frecuencias alélicas de una población), facilita la creación de bancos de recursos genéticos de razas en peligro de extinción, promueve la comercialización de embriones entre países y elimina las patologías asociadas al mantenimiento de animales vivos.

En la actualidad, hay dos métodos de criopreservación disponibles para embriones: la congelación lenta o convencional y la vitrificación. Las principales diferencias entre estos dos métodos son la concentración de los crioprotectores y las velocidades de enfriamiento (Vajta y Kuwayama, 2006). Sin embargo, tanto la congelación lenta como la vitrificación pueden afectar de diferente manera la fisiología y la supervivencia del embrión (Fig. 1.5). A pesar de los avances en las técnicas de criopreservación, los resultados continúan siendo distintos entre embriones bovinos producidos *in vitro* y embriones *in vivo*. Los medios utilizados en el CIV, la calidad del embrión y el estadío embrionario influyen directamente en la supervivencia de los embriones a la criopreservación. Por lo tanto, las investigaciones para mejorar los métodos de criopreservación en embriones bovinos producidos *in vitro* continúan.

1.3.1. Congelación lenta

En la congelación lenta, los embriones alcanzan un equilibrio osmótico en un medio con crioprotectores en bajas concentraciones. En general, el procedimiento de congelación lenta para embriones bovinos implica enfriar y estabilizar el embrión entre -5.5 y -7° C, inducir la cristalización (seeding) y disminuir la temperatura hasta temperaturas próximas a -32 °C a una velocidad de 0.3 - 0.6 °C por minuto para posteriormente almacenarlos en nitrógeno líquido a -196 °C (Hasler, 2007; Almiñana y Cuello, 2015).

Una de las mayores ventajas de la congelación lenta en comparación con la vitrificación es que simplifica la transferencia directa (TD) de embriones a receptoras, siendo el método de criopreservación predilecto para embriones

bovinos *in vivo* (Hasler *y cols.,* 1995; Viana *y cols.,* 2017). Por lo general se tiende a asumir que los embriones bovinos producidos *in vitro* son más sensibles a la congelación lenta y producen menores indices de gestación en comparación a la técnica de vitrificación. Sin embargo, por los mejores índices de gestación que se obtienen, más del 70% de los embriones bovinos producidos *in vitro* a escala mundial han sido transferidos en fresco (Perry, 2016). Aunque, recientemente, se han desarrollado sistemas eficientes de congelación lenta y TD para embriones bovinos producidos *in vitro* (Sanches *y cols.,* 2016), que son usados principalmente por empresas comerciales.

El proceso de congelación lenta se requiere un equipo relativamente costoso para controlar las curvas de enfriamiento, pero permite una gestión más rápida cuando se dispone de un número elevado de embriones. Además, la congelación lenta posibilita la TD simple de realizar en campo, satisface las exigencias sanitarias y facilita ampliamente la comercialización mundial de embriones.

1.3.2. Vitrificación

La vitrificación implica la solidificación de una solución de base acuosa para alcanzar un estado vítreo, amorfo, carente de cristales de hielo. Durante el proceso de vitrificación, el embrión se expone primero a una solución de crioprotectores menos concentrada (1-1.5 mol L^{-1}), después a una solución de crioprotectores más concentrada (4-8 mol L^{-1}) y luego se enfría a velocidad ultra-rápida (≈-20.000 °C por minuto o superior), por lo general usando

nitrógeno líquido (-196 °C). Existen diferentes dispositivos para manejar y alojar el embrión durante la vitrificación (Rall y Fahy, 1985; Arav, 2014).

Desde su desarrollo (Rall y Fahy, 1985), la vitrificación se ha convertido en el método de criopreservación más utilizado para embriones bovinos PIV. La vitrificación es un procedimiento simple, no requiere equipos costosos, es barata (Marco-Jiménez *y cols.,* 2016) y cuando el número de embriones es reducido se puede realizar en poco tiempo. Sin embargo, algunos protocolos de vitrificación producen toxicidad por la exposición a altas concentraciones de crioprotectores y un mayor daño causado por el choque osmótico. Para tratar de disminuir la toxicidad específica de cada crioprotector, se ha tratado de encontrar crioprotectores menos tóxicos y más permeables, y usar dos o tres crioprotectores añadidos de forma gradual (Saragusty y Arav, 2011; Trigal *y cols.,* 2012; Caamaño *y cols.,* 2015). La desventaja principal del método de vitrificación es que no se ha desarrollado un método eficaz que permita la TD a receptoras y por tanto es necesario contar con personal entrenado para manipular y evaluar los embriones antes de la transferencia, dificultando su aplicación comercial a gran escala.

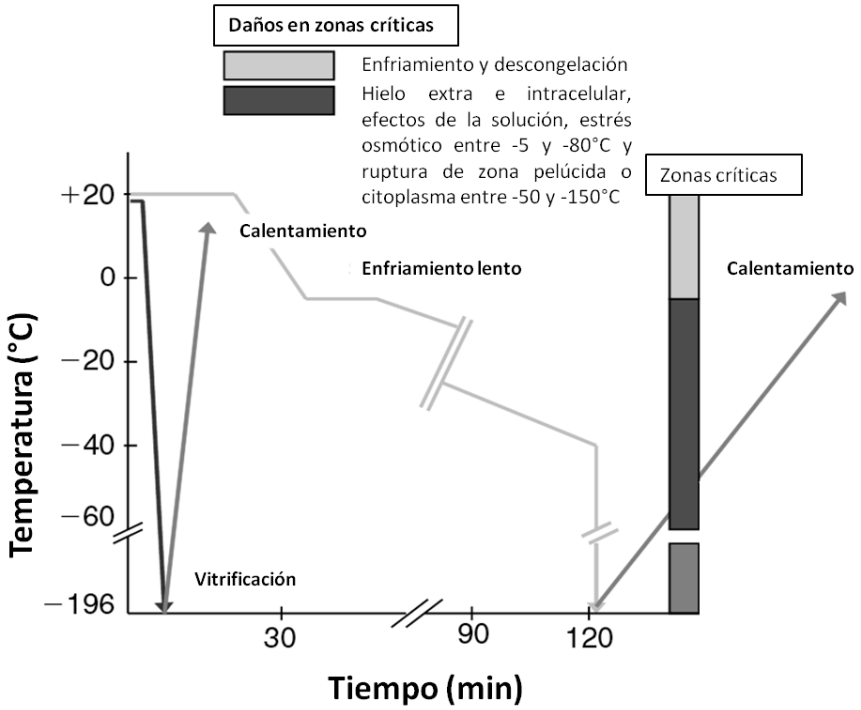

Fig. 1.5. Esquema de enfriamiento y zonas críticas de daño celular durante la criopreservación. Adaptado de Quin, 2010 y Vatja, 2006.

1.4. Evaluación de la calidad y viabilidad de embriones bovinos producidos *in vitro*

En general, la calidad y los porcentajes de desarrollo de embriones bovinos producidos *in vitro* pueden variar ampliamente. Estas diferencias vienen dadas por la diversidad inherente al potencial de los gametos y por las condiciones de los medios de producción in vitro de embriones. Uno de los objetivos principales de la investigación en embriones bovinos *in vitro* es desarrollar un método de selección eficaz que permita predecir con precisión la calidad y viabilidad de los embriones. Los métodos que se han utilizado para este objetivo se clasifican como invasivos o no invasivos. Desde el punto de vista práctico, se prefiere métodos no invasivos ya que las técnicas invasivas, por lo general, implican un daño parcial en la estructura (reduce la viabilidad) o la muerte del embrión.

1.4.1. Morfología

La evaluación morfológica de los embriones bovinos se ha estudiado ampliamente, primero en embriones *in vivo* y posteriormente en embriones *in vitro*, ambos con el objetivo de seleccionar con precisión los mejores embriones, tanto en fresco como después de criopreservación. Los parámetros de morfología en embriones bovinos *in vivo* incluyen la forma, el color, el estadío del embrión, el número de células extruidas y degeneradas y el número y tamaño de las vesículas (Linder y Wright 1983; Stringfellow y Givens, 2010); parámetros que también se evalúan en embriones bovinos PIV (Hasler, 1995; Van Soom *y cols.,* 1997).

En la observación mediante estereomicroscopio (Fig. 1.6), los embriones producidos *in vitro* muestran morfología alterada, diferentes patrones de desarrollo, mayor número de vacuolas y parecen ser más opacos en comparación con los embriones obtenidos *in vivo* (Iwasaki *y cols.,* 1990; Rizos *y cols.,* 2002). Los blastocistos bovinos producidos *in vitro* además presentan uniones intercelulares más débiles que los blastocistos *in vivo* (Prather, 1993). Estas diferencias morfológicas entre embriones *in vivo* e *in vitro* también han sido descritas mediante microscopía electrónica (Crosier *y cols.,* 2000; Crosier *y cols.,* 2001).

Las condiciones de CIV también pueden afectar a la morfología del embrión en desarrollo. Por ejemplo, la suplementación del medio de cultivo con suero o diferentes tipos de BSA altera el aspecto de los embriones. Sin embargo, los blastocistos bovinos *in vitro* producidos sin suero tienen una morfología más parecida a los blastocistos *in vivo* (Rizos *y cols.,* 2003). Por lo general, el uso

de suero en el CIV aumenta los niveles de lípidos en el embrión. Los lípidos incorporados del suero pasan a formar parte de las vesículas formadas en los embriones, lo que hace que el embrión adopte una apariencia más oscura y con frecuencia vesicular o punteada (Gardner, 1994). Además, el suero puede contener sustancias que promueven la síntesis de lípidos por el embrión.

Además, es factible que los suplementos de albúmina también puedan alterar la morfología del embrión. Existe una amplia variedad de preparaciones de BSA comerciales, aunque la gran mayoría contiene componentes no conocidos de bajo peso molecular (Francis *y cols.,* 2010), entre los cuales la parte lipídica puede variar. Por lo tanto, diferentes fuentes de albúmina podrían también modificar el aspecto del embrión.

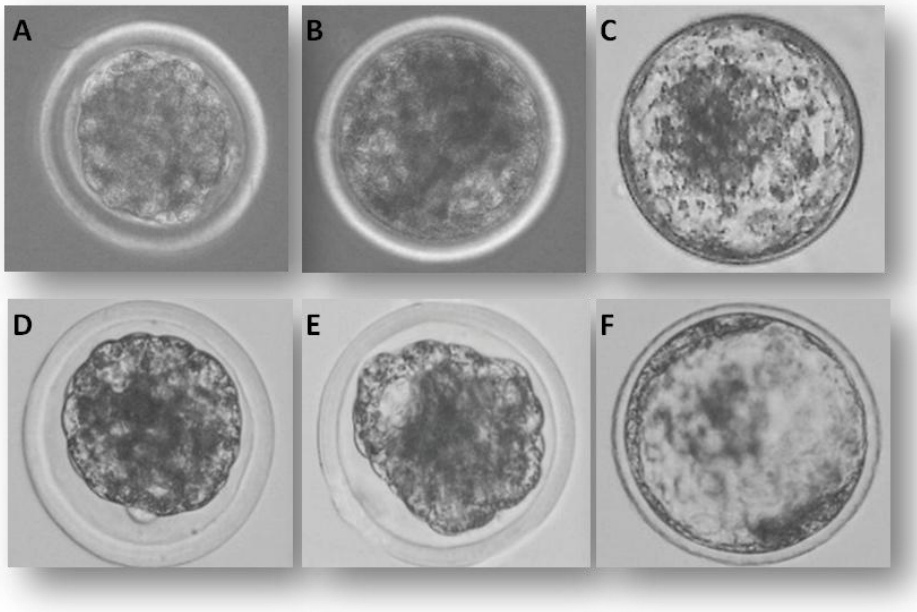

Fig. 1.6. Diferentes estadios de embriones bovinos producidos *in vitro* (**A,B,C**) e *in vivo* (**D,E,F**). **A,D:** Mórula; **B,E:** Blastocisto temprano; **C,F:** Blastocisto expandido. (**D,E,F:** Adaptado de Jahnke *y cols.,* 2014)

1.4.2. Recuento total y diferencial de células embrionarias

La evaluación del número total de células embrionarias se considera una prueba de calidad embrionaria. Para realizar un recuento celular total generalmente se usan tinciones de ADN que ayudan a visualizar los núcleos celulares. Las tinciones fluorescentes de ADN, Bisbenzimida (Bb) y DAPI, tiñen los núcleos en azul y son las más usadas para evaluar el número total de células embrionarias. La membrana celular es permeable a la Bb y DAPI, por lo que no es necesario permeabilizar el embrión previamente.

Otro colorante fluorescente de ADN usado regularmente es el yoduro de propidio (IP), que tiñe los núcleos celulares en rojo. El IP es impermeable a las membranas celulares, por lo cual el embrión debe ser permeabilizado previamente. Así, Thouas *y cols.,* (2001) desarrollaron una técnica, no inmunohistoquímica (en contraste con Van Soom *y cols.,* 2001), que hace posible el marcaje fluorescente de los dos tipos celulares del embrión. El IP puede usarse en combinación con Bb o DAPI para realizar un marcaje diferencial entre las células de la MCI y del TF. Tras una primera permeabilización en una solución de IP y tritón los embriones se fijan en etanol con Bb. Como resultado se distinguen las células de la MCI, situadas internamente y teñidas de azul, por efecto de la Bb, y las células del TF de color rosa debido a la combinación de IP y Bb.

Una posible asincronía en la división mitótica de los blastómeros podría reflejarse en alteraciones de compactación de la mórula y en la posterior diferenciación de la MCI y del TF (Johnson y Ziomek, 1981). La tinción diferencial se puede utilizar para obtener una mejor estimación de la calidad y

del grado de diferenciación del embrión. Por lo tanto, esta técnica permite comparar el desarrollo de MCI y TF en diferentes condiciones de cultivo para evidenciar desviaciones en el desarrollo de embriones (Van Soom *y cols.,* 2001).

El porcentaje de células de la MCI respecto al número total de células embrionarias se ha considerado indicador de calidad, y esa relación es menor cuando los embriones bovinos son producidos *in vitro* (Iwasaki *y cols.,* 1990). Los embriones producidos *in vitro* presentan un número de células totales comparable a los producidos *in vivo*, aunque, su número de células de la MCI es significativamente inferior (Van Soom *y cols.,* 2001). La producción de embriones con más células en la MCI puede mejorar los índices de gestación (Loureiro *y cols.,* 2009); por el contrario, un exceso de células del TF puede conducir a anomalías de la gestación (Van Soom *y cols.,* 1997).

También se han descrito que algunas moléculas como por ejemplo el ácido retinoico (Rodríguez *y cols.,* 2006; 2007), la activina A (Trigal *y cols.,* 2011), el suero (Mucci *y cols.,* 2006), la forskolina (Sudano *y cols.,* 2017) influyen en la diferenciación del blastocisto bovino PIV. Por otro lado, los embriones cultivados en un medio libre de BSA no mostraron cambios significativos en los recuentos celulares ni tampoco índices de gestación más elevados que los embriones cultivados con BSA (Lim *y cols.,* 2007). Sin embargo, aunque se precisa un número mínimo de células en el blastocisto para establecer preñez (Iwasaki *y cols.,* 1990), el número óptimo de células y cómo han de distribuirse entre la MCI y el TF no están claros.

1.4.3. Apoptosis y daño celular

La apoptosis es un proceso natural que tiene lugar en todos los tipos de células. Su función durante el desarrollo del embrión es eliminar las células anormales, no funcionales o potencialmente peligrosas. La incidencia de apoptosis o muerte celular programada es un parámetro importante para evaluar la calidad embrionaria. Un aumento desmesurado en la proporción de células apoptóticas puede conducir a la muerte del embrión (Levy *y cols.,* 2001; Fabian *y cols*., 2005). También se ha descrito la necrosis celular, que es un tipo de muerte celular accidental debido a factores externos y que se puede diferenciar de la apoptosis por el aspecto de las células necróticas (Majno y Joris, 1995).

Los métodos más comunes para la detección de apoptosis están basados en técnicas de tinción inmunológicas. La prueba TUNEL (*Terminal deoxynucleotidyl transferase dUTP nick end labeling*) se basa en el uso de la deoxinucleotidil transferasa terminal, enzima que cataliza la unión de dUTP marcado con fluoresceína, a los extremos 3'-OH libres presentes en el DNA fragmentado como consecuencia de la apoptosis. Se consideran apoptóticos aquellos núcleos que presentan un marcaje positivo y una morfología particular, según criterios descritos por Gjorret *y cols.,* (2003).

La cuantificación de la apoptosis ha recibido especial atención debido a su correlación con la muerte embrionaria temprana, condiciones de desarrollo subóptimas, respuestas celulares al estrés y reducción de la viabilidad embrionaria (Byrne *y cols.,* 1999; Matwee *y cols.,* 2000; Gardner *y cols.,* 2000; Rodríguez *y cols.,* 2006). Además, los índices de apoptosis en células

embrionarias muestran alta correlación con la supervivencia a la criopreservación de embriones bovinos producidos *in vitro* (Sudano *y cols.,* 2012).

1.4.4. Contenido de lípidos intracelulares

Los lípidos son moléculas hidrofóbicas que desempeñan papeles fundamentales en el metabolismo embrionario de los mamíferos. Son fuente de energía, mediadores de señalización celular y base estructural de las membranas plasmáticas (McKeegan y Sturmey, 2011). El contenido de lípidos en las células embrionarias se organiza en gotas de diferentes tamaños. Cada gota lipídica contiene un núcleo de lípidos esterificados, como son pueden ser los triacilglicéridos, los esteres de colesterol, los esteres de retinol y también ácidos grasos libres (Fig.1.7). Cada gota está recubierta por una monocapa de fosfolípidos y proteínas específicas entre las que destacan la familia de las perilipinas (Guo *y cols.,* 2009) (Fig.1.6). La tinción fluorescente *Nile Red* (NR) se usa frecuentemente para visualizar gotas de lípidos intracelulares en ovocitos y embriones de mamíferos (Genicot *y cols.,* 2005; Leroy *y cols.,* 2005). En un ambiente hidrofóbico, el NR emite una fluorescencia que va de amarillo a naranja. Los lípidos neutros como los triglicéridos (gotas de lípidos) son fluorescentes en amarillo, mientras que los lípidos polares (fosfolípidos) son fluorescentes en el espectro naranja. La fluorescencia emitida por el NR es específica para las gotas de lípidos y no para el citosol o el compartimento nuclear, debido a que las gotas de lípidos contienen principalmente triglicéridos (Genicot *y cols.,* 2005).

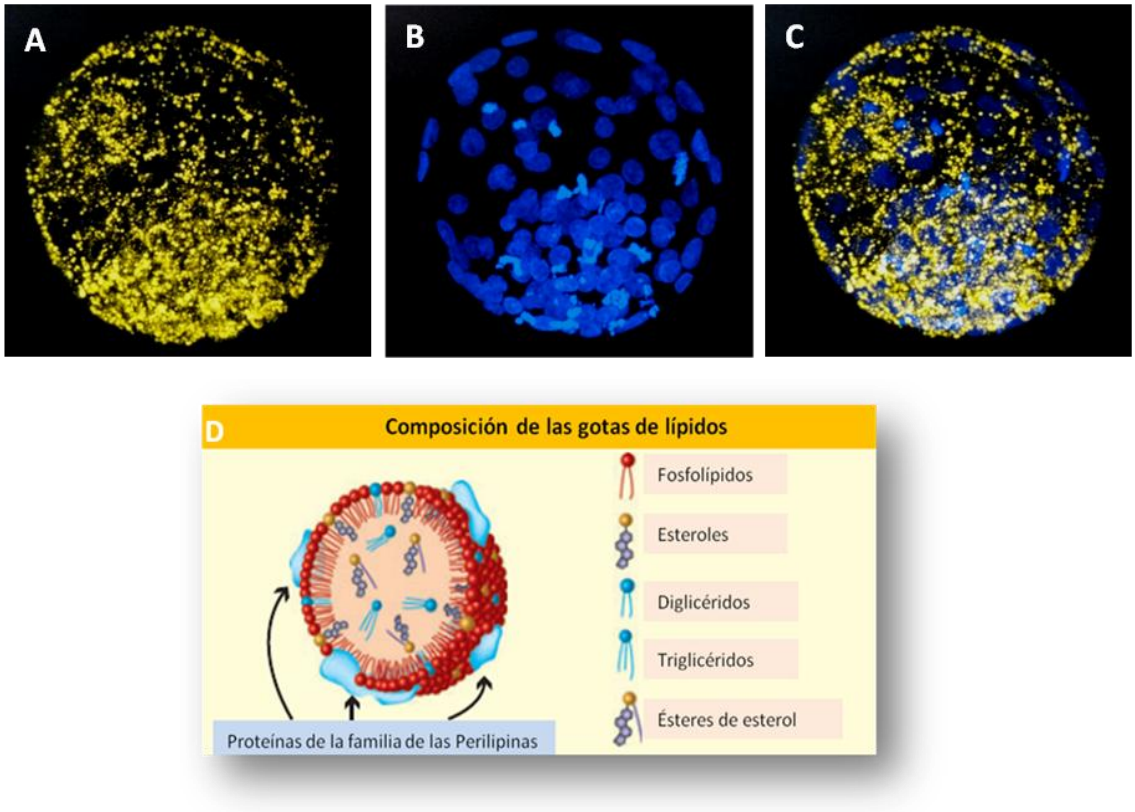

Fig. 1.7. Acumulación de lípidos intracelulares en un blastocisto expandido bovino producido *in vitro*. **A:** gotas de lípidos intracelulares teñidas con Nile Red. **B:** núcleos celulares teñidos con DAPI. **C:** imagen conjunta de gotas de lípidos intracelulares y núcleos celulares **D:** Representación esquemática de la composición de las gotas de lípidos (Adaptado de Guo *y cols.,* 2009).

Durante periodos de deficiencia energética, la célula accede a los depósitos lipídicos y recupera la energía almacenada mediante la actividad de hidrolasas especificas, conocidas como lipasas (Ducharme y Bickel, 2008). Los lípidos usan principalmente la ruta de la β-oxidación mitocondrial para generar ATP durante la maduración de los ovocitos y el desarrollo embrionario temprano (Sturmey y Leese, 2003; Dunning *y cols.,* 2010), aunque también pueden usar el peroxisoma (oxidasas, catalasas).

El exceso de lípidos intracelulares aumenta la sensibilidad del embrión al estrés oxidativo y reduce la supervivencia a la criopreservación de embriones bovinos producidos *in vitro* (Abe *y cols.,* 2002, Reis *y cols.,* 2003, Sudano *y cols.,* 2011).

Se ha descrito que la acumulación de lípidos puede verse influenciada por el uso de ciertos suplementos (por ejemplo suero, BSA) durante el CIV o como resultado de alteraciones en el metabolismo de la energía, resultando también afectadas las propiedades y la estabilidad de las membranas celulares (Dinnyes y Nedambale, 2009).

1.4.5. Prueba de supervivencia a la criopreservación

Los procesos de criopreservación son una fuente de estrés para los embriones bovinos (Puscheck *y cols.,* 2015) por lo que la supervivencia a estos procesos puede ser usada como indicador de la calidad embrionaria. En la prueba de supervivencia a la criopreservación *in vitro* se evalúan los porcentajes de re-expansión y de eclosión de los embriones después de un período de CIV (Dinnyés *y cols.,* 1999). Varios sistemas de cultivo se han utilizado para este fin y la mayoría bajo condiciones no definidas (Massip *y cols.,* 1993; Rizos *y cols.,* 2001).

El estadío de desarrollo y la morfología son los parámetros habitualmente usados para seleccionar los embriones *in vitro* que van a ser criopreservados. Esto es así en la práctica, a pesar que la estimación de la supervivencia a la criopreservación sigue siendo incierta y subjetiva (Sudano *y cols.,* 2012). La supervivencia a la criopreservación puede verse afectada por el sistema de cultivo utilizado antes y después de la criopreservación en los embriones bovinos PIV (Kaidi *y cols.,* 1998; Rizos *y cols.,* 2001). Además, la intensidad del daño causado por la criopreservación a la célula embrionaria depende del

tamaño y forma de éstas, así como de la permeabilidad de la membrana y la calidad de los embriones (Vatja *y cols.,* 2006).

La presencia de suero durante el CIV es la principal causa del aumento en el contenido lipídico de embriones bovinos *in vitro* (Abe *y cols.,* 2002), lo que incide en su baja supervivencia a la criopreservación (Mucci *y cols.,* 2002; Rizos *y cols.,* 2003). En consecuencia, se han estudiado varias estrategias para tratar de reducir el contenido de lípidos en los embriones.

Sudano y cols., (2011) demostraron que una concentración reducida de suero (2.5% v/v) y la adición de eosulfato de fenazina al CIV de embriones bovinos, inhibe la síntesis de ácidos grasos y mejora la supervivencia a la criopreservación. En otro estudio Gómez *y cols.,* (2008) establecieron que los blastocistos PIV cultivados en medio SOF con BSA pero sin suero mostraron una mayor tasa de supervivencia a la vitrificación frente a embriones cultivados en SOF con suero. Del mismo modo, se ha descrito que el tratamiento con forskolina antes de la vitrificación mejora la supervivencia a la criopreservación de embriones bovinos (*Bos indicus*) producidos *in vitro,* obteniéndose mejores índices de gestación después de la transferencia a receptoras (Sánchez *y cols.,* 2013).

1.4.6. Expresión génica

El transcriptoma se define como el conjunto completo de moléculas de ARN expresadas en una célula u organismo durante una etapa específica del desarrollo o condición fisiológica. Por lo tanto, el análisis de los patrones de expresión de genes que pueden estar implicados en la traducción de proteínas

esenciales en el desarrollo embrionario proporciona información valiosa sobre los posibles efectos ejercidos por factores ambientales sobre el embrión temprano (Cagnone *y cols.,* 2012; Dufort *y cols.,* 2015). Las proteínas que traducen los genes expresados durante el desarrollo embrionario están implicadas en diversos procesos biológicos que incluyen metabolismo, señales de GFs y citoquinas, adaptación al estrés, transcripción y traducción, regulación epigenética de la transcripción, apoptosis, compactación y formación de blastocisto (Wrenzycki *y cols.,* 2005; Ross y Chitwood, 2012).

El medio de cultivo de embriones después de la FIV puede tener un efecto drástico sobre el patrón de expresión génica en el embrión, lo que a su vez afectará al desarrollo normal del blastocisto. Así, se han evidenciado cambios en los patrones de expresión génica no solo cuando se comparan sistemas de cultivo *in vivo* e *in vitro*, sino también entre diferentes sistemas de CIV (Eckert y Niemann, 1998; Wrenzycki *y cols.,* 2001; Rizos *y cols.,* 2002; Lonergan *y cols.,* 2003; Cagnone *y cols.,* 2012; Van der Weijden *y cols.,* 2017).

La sensibilidad de los análisis de expresión génica han mejorado mucho en los últimos años, y hoy en día diferentes técnicas transcriptómicas permiten analizar la expresión génica en ovocitos y embriones bovinos o incluso en blastómeros individuales (Chitwood *y cols.,* 2013; Jiang *y cols.,* 2014).

1.4.7. Dimorfismo sexual

Los embriones bovinos macho y hembra difieren no solo en los cromosomas sexuales, sino también en la expresión génica de genes presentes en los cromosomas autosómicos (Bermejo-Álvarez *y cols.,* 2010; Lowe *y cols.,* 2015),

la configuración epigenética (Bermejo-Álvarez *y cols.,* 2008; Dobbs *y cols.,* 2013), la regulación proteómica (Gómez *y cols.,* 2013) y en los perfiles metabolomicos (Muñoz *y cols.,* 2013; Gómez *y cols.,* 2016; Gómez *y cols.,* 2017; Gómez *y cols.,* 2018).

En los mamíferos, los cambios en el entorno materno durante el período preimplantacional afectan de manera diferente a embriones machos y hembras, lo cual también se evidencia en los fenotipos de recién nacidos y adultos (Hansen *y cols.,* 2016). La proporción de embriones macho/hembra puede ser modificada por factores ambientales tales como la dieta materna *in vivo* y la composición de los medios de cultivo de embriones *in vitro*. Los embriones macho y embriones hembra difieren en su fisiología durante los estadíos de desarrollo previos a la implantación (Gardner *y cols.,* 2010). De igual manera, el microambiente del medio de cultivo, los niveles de nutrientes, el tipo de suplemento proteico y la concentración de oxígeno pueden afectar al desarrollo embrionario de manera específica dependiendo del sexo del embrión (Wale y Gardner, 2015).

Durante el período preimplantacional los embriones macho y hembra son genéticamente idénticos en sus cromosomas autosómicos, aunque la regulación transcripcional es específica para cada sexo. En bovino, durante la primera división celular, los embriones macho se dividen antes que los embriones hembra (Gutiérrez-Adán, 1996; Pérez-Cerezales *y cols.,* 2018) y el dimorfismo sexual continua siendo apreciable en el estadío de mórula (Denicol *y cols.,* 2015). En el estadío de blastocisto, un tercio de todos los genes que se expresan difieren entre embriones macho y hembra, existiendo una mayor expresión de genes ligados al cromosoma X en embriones femeninos

(Bermejo-Álvarez *y cols.,* 2010). En algunas condiciones de CIV, se han señalado diferencias dependientes del sexo del embrión, como la velocidad de desarrollo embrionario, la supervivencia después de la vitrificación, el número de células en la etapa de blastocito, los porcentajes de apoptosis y el metabolismo (Gutiérrez-Adán *y cols.,* 2006; Ghys *y cols.,* 2016; Gómez *y cols.,* 2018).

Entre las diferencias metabólicas específicas del sexo del embrión, el metabolismo de la glucosa es una de las más estudiadas, particularmente con respecto a la vía de la pentosa fosfato (Kimura *y cols.,* 2005; Sturmey *y cols.,* 2010). En bovino, el aumento de la expresión de la *glucosa 6-fosfato deshidrogenasa* (*G6PD*) en blastocistos hembra (Gutiérrez-Adán, *y cols.,* 2000, Wrenzycki, *y cols.,* 2002) y de *SLC2A3* en blastocistos macho (Morton, *y cols.,* 2007), provoca diferencias en el metabolismo de la glucosa entre embriones de ambos sexos. Sin embargo, el metabolismo de la glucosa no destacó como una vía sexualmente dimórfica en un estudio de Bermejo Álvarez *y cols.,* (2010a) sobre las diferencias globales de transcritos de blastocistos bovinos macho y hembra. Así, el análisis de transcritos específicos implicados en la glucólisis anaerobia o la vía de la pentosa fosfato no mostró una desequilibrio claro en la proporción de sexos (Bermejo Álvarez *y cols.,* 2011a). Además, Cagnone *y cols.,* (2011) señalaron que la respuesta transcripcional de blastocistos bovinos a la presencia de glucosa no está relacionada con el sexo. De igual manera, Gómez *y cols.,* 2013 indicaron que la concentración de D-fructosa aumentó en el fluido uterino (FU) bovino en presencia de embriones PIV hembra, comparado con el FU con embriones PIV macho, mientras que los

niveles de D-glucosa fueron similares. Por lo tanto, el metabolismo de la glucosa asociado al dimorfismo sexual no está exento de controversias.

Por otro lado, en un análisis global de expresión génica de blastocistos bovinos PIV, la actividad de la mitocondria y la traducción de proteínas, la proteólisis y el transporte de proteína se encontraron afectadas por el sexo del embrión (Bermejo Álvarez *y cols.,* 2010). Además, los embriones macho y hembra difieren en el ADN mitocondrial (Bermejo Álvarez *y cols.,* 2008) y el metabolismo de aminoácidos (Sturmey *y cols.,* 2010). Las diferencias específicas por sexo en estas y otras rutas (implicadas en la apoptosis y la comunicación materno embrionaria) proporcionan una base molecular para dar respuestas específicas del sexo a deficiencias nutricionales, y también pueden utilizarse para desarrollar métodos no invasivos de diagnóstico del sexo de los embriones (Gómez *y cols*., 2016; Sturmey *y cols.,* 2010).

En un estudio reciente, Heras *y cols.,* (2016) demostraron que las condiciones subóptimas de CIV pueden tener un impacto diferente en los embriones de acuerdo con su sexo. Así, los embriones macho PIV tienen tres veces más genes expresados diferencialmente comparados con embriones *in vivo* y con embriones hembra PIV. Además, el útero de la vaca proporciona a los embriones macho un entorno más embriotrófico que a los embriones hembra (Gómez *y cols.,* 2013). Por lo tanto, el estudio del dimorfismo sexual puede ayudar a diseñar medios de CIV adaptados al sexo del embrión.

1.5. La viabilidad del embrión en las receptoras

1.5.1. Establecimiento de la gestación

Los índices de gestación después de la transferencia son un muy buen indicador de la viabilidad de los embriones bovinos PIV. Por lo general, los embriones PIV se transfieren usando la vía transcervical -no invasiva- al igual que los embriones recogidos *in vivo*. La edad y estadío del embrión *in vitro* junto con la fase del ciclo estral de la receptora se deben sincronizar estrechamente (Hasler *y cols.,* 2014; Randi *y cols.,* 2015)

En bovinos, el período embrionario discurre entre la fecundación y el final de la organogénesis -día 42 de gestación-, a partir del cual se inicia el período fetal. En la luz del útero el blastocisto eclosionado se elonga antes de implantarse, adquiriendo sucesivamente formas esférica, ovoide y filamentosa. La elongación del blastocisto coincide con la gastrulación (diferenciación de capas germinativas) y el embrión se denomina conceptus. El establecimiento de la gestación implica el crecimiento y elongación del conceptus, su reconocimiento por el útero, la implantación y la placentación (Spencer *y cols.,* 2008).

La elongación está controlada por la progesterona (P4), que actúa regulando el crecimiento del TF (Spencer *y cols.,* 2007). El conceptus crece dentro de la luz uterina del cuerno ipsilateral al cuerpo lúteo, espacio que ocupa por completo hacia el día 17 de gestación. Durante la elongación el conceptus desarrolla una capacidad creciente para secretar interferón-tau (IFN-τ; Kubisch, 1998; Bertolini y cols., 2002), el cual modula la síntesis de prostaglandinas en el endometrio para bloquear los pulsos luteolíticos de la prostaglandina $PGF_2\alpha$ (Spencer *y cols.,* 2007) y para favorece la secreción de prostaglandina E2 (Sponchiado *y*

cols., 2017). La producción de IFN-τ, que se realiza en las células del TF (Spencer y Bazer, 2004; Robinson *y cols.,* 2008), es la señal para el reconocimiento materno de la gestación y alcanza su nivel máximo entre los días 15 y 17 de gestación (Wolf *y cols.,* 2003).

Se ha descrito ampliamente que la expresión de IFN-τ es evidente tan pronto como se desarrollan las células del TF en los estadíos de morula tardía y blastocisto temprano en embriones bovinos producidos *in vitro* e *in vivo* (Hernández-Ledezma *y cols.,* 1993; Kubisch *y cols.,* 1998; Bertolini *y cols.,* 2002; Lonergan *y cols.,* 2003). Aunque, los blastocistos bovinos de día 7 producidos *in vivo* o *in vitro* producen cantidades muy bajas de IFN-τ (Kubisch *y cols.,* 1998), se ha demostrado recientemente que el blastocisto de día 7 (no eclosionado) es capaz de estimular la expresión de genes inducidos por el IFN-τ en la unión uterotubal de la vaca gestante (Sponchiado *y cols.,* 2017).

El éxito de la gestación en el ganado bovino precisa de funciones endometriales tempranas dependientes e independientes del embrión. Así, la proximidad física del embrión regula la función endometrial de manera paracrina, en tanto que la regulación del transcriptoma endometrial independiente del embrión puede favorecer etapas posteriores del desarrollo embrionario, tales como la elongación y la implantación (Sponchiado *y cols.,* 2017). La implantación del conceptus en el útero comienza aproximadamente en el día 19 de la gestación y en el día 20 se desarrollan las primeras vellosidades placentarias. Alrededor del día 27 el entramado de vellosidades situados frente a las carúnculas uterinas da forma los primeros cotiledones.

Actualmente en bovinos, la mayoría de diagnósticos de gestación se llevan a cabo mediante ultrasonografía transrectal del tracto reproductivo para detectar la presencia o ausencia del embrión generalmente a partir del día 27 o 28. Además, la ultrasonografía transrectal permite evaluar la dinámica folicular, la morfología del cuerpo lúteo y la viabilidad embrionaria ó fetal, entre otras aplicaciones (Ginther, 2014).

Los embriones bovinos PIV producen menores índices de gestación que los embriones producidos *in vivo* mediante MOET (Pontes *et al*., 2009; Siqueira *et al*., 2009). Así, los índices de gestación después de la transferencia de embriones bovinos PIV son entre 10% y 30% más bajos que las gestaciones a partir de embriones *in vivo* (de Sousa *y cols.,* 2017; Pontes *y cols.,* 2009; Rasmussen *y cols.,* 2013). Los índices de gestación obtenidos después de la transferencia de embriones dependen tanto de la receptividad de la receptora como de la viabilidad del embrión. En este sentido, se han reportado índices de gestación de embriones bovinos PIV significativamente menores en vacas (33.1%) que en novillas (44.0%) (Aoki *y cols.,* 2004). Además, los embriones bovinos producidos *in vitro* transferidos en vacas de primer parto también producen menores índices de concepción (39.6%) que las novillas (47.5%) (van Wagtendonk-de Leeuw *y cols.,* 1997).

1.5.2. Pérdidas embrionarias tempranas y aborto

La mayoría de las pérdidas de embriones ocurren entre el día 8 y el día 16 de gestación coincidiendo con el reconocimiento materno del embrión. El aborto es la interrupción de la gestación después de la organogénesis, antes de que el

feto expulsado pueda sobrevivir. Si la gestación termina antes de que se complete la organogénesis entre la fecundación y el día 42 de gestación, se denomina muerte embrionaria temprana (Walsh *y cols.,* 2011), mientras que la expulsión de fetos sin vida desde el día 42 hasta el final de gestación se denomina aborto. La mortalidad fetal a término se caracteriza por la evidencia de un ternero muerto sin que sus pulmones hayan llegado a insuflarse.

En bovinos, una de las principales causas del bajo éxito reproductivo es la mortalidad embrionaria temprana (Santos *y cols.,* 2004). Existe una pérdida significativa de gestaciones tras la transferencia de embriones *in vitro* que principalmente se produce durante las etapas más tempranas de la gestación. Los mayores porcentajes de pérdidas embrionarias en la gestación bovina ocurren hasta día 42 (final de la organogénesis) en comparación con el período fetal o el período perinatal (desde el parto hasta el día 28 posparto) (Farin *y cols.,* 2001, 2006).

Sreenan y Diskin (1986) calcularon un índice de mortalidad embrionaria y fetal del 40% para vacas con producciones moderadas de leche, donde el 75% de las pérdidas embrionarias fueron entre los días 8 y 16 después de la inseminación artificial (IA). En sistemas de producción de leche en pastoreo, tras la IA, los índices de pérdidas embrionarias y fetales evaluados entre los días 28 y 84 de gestación fueron similares para vacas leche (7,2%) y novillas (6,1%). Además, un 47.5% de las pérdidas embrionarias totales ocurrieron entre los días 28 y 42 de gestación (Silke *y cols.,* 2001).

Después de la transferencia de embriones bovinos PIV se han señalado porcentajes del 45% de pérdidas embrionarias en día 14, coincidiendo con el

período de reconocimiento materno de la gestación (McMillan *y cols.,* 1997; Mamo *y cols.,* 2011). Un análisis retrospectivo de 2.300 embriones PIV transferidos a receptoras reveló una tasa de supervivencia promedio del 50% al día 24, del 40% al día 60 y del 30% al término de la gestación (Peterson y Lee, 2003). Además, Muñoz *y cols.,* (2014), después de la transferencia de embriones PIV frescos, obtuvieron 59% de gestaciones a día 60 y 52% de nacimientos, mientras que, con embriones PIV vitrificados señalaron 36% de gestaciones a día 60 y 31% de nacimientos. Es importante destacar que las mayores proporciones de pérdidas embrionarias y fetales varían con los medios y sistemas de CIV utilizados (Farin *y cols.,* 2006).

Aunque los abortos son menos abundantes que las pérdidas embrionarias tempranas, aquellos causan perjuicios económicos graves, particularmente en rebaños de parto estacional. La interrupción tardía de la gestación impide a menudo reinsertar a tiempo las vacas en programas de reproducción, lo cual aumenta las tasas de sacrificio (Diskin *y cols.,* 2012).

1.5.3. Resultados perinatales con embriones producidos *in vitro*

Las anormalidades más excepcionales observadas en el período perinatal después de la transferencia de embriones PIV es la aparición de terneros de mayor peso al nacimiento en comparación con los embriones producidos *in vivo*. Los elevados porcentajes de muerte perinatal asociados a las gestaciones de embriones PIV son atribuibles en gran medida a la distocia provocada por fetos de gran tamaño durante el parto (Behboodi, 1995; Farin, 2006).

Los terneros nacidos a partir de la transferencia individual de embriones frescos producidos *in vitro* presentan mayores pesos al parto, y períodos de gestación más largos, que los terneros de IA y ET gemelares (Sinclair, 1995). Se ha descrito un elevado peso al parto asociado a embriones producidos *in vitro* en varias razas bovinas tanto en *Bos taurus* como en *Bos indicus* (Kruip y Den Daas, 1997; van Wagtendonk-de Leeuw 1998; McEvoy, 1998; Numabe 2000; Bertolini 2002). Así, en Holstein, más del 34% de los terneros producidos *in vitro* pueden pesar más de 50 kg al parto (Kruip y Den Daas, 1997; Lazzari, 2002). En Gyr, la transferencia de embriones frescos PIV dio como resultado un mayor peso al nacer que los controles de IA, efecto más acusado en los terneros machos (Camargo, 2010).

Tras la gestación de embriones bovinos PIV, el mayor peso al nacimiento de los terneros refleja una desproporción orgánica (Farin, 1995; Bertolini, 2004). Curiosamente, los terneros de embriones frescos PIV mostraron no solo mayores pesos al nacer, sino también concentraciones elevadas de fructosa en plasma después del nacimiento en comparación con los controles *in vivo* (Bertolini, 2004).

La facilidad de parto depende de la duración de la gestación y del peso de los terneros al parto, y se ve afectada por el sexo fetal, el fenotipo de los progenitores, la nutrición materna, el ambiente durante el último trimestre de gestación y el medio de CIV (Mee, 2008).

1.5.4. Salud perinatal de terneros producidos *in vitro*

Los factores de riesgo prenatales pueden afectar negativamente algunos rasgos de la progenie. Por ejemplo, la nutrición subóptima de los progenitores, el estrés gestacional, la exposición a sustancias químicas y las tecnologías reproductivas usadas en los programas de mejora genética inciden en el crecimiento posparto, la eficiencia alimenticia, la producción de leche, la composición de la canal, el bienestar animal y el potencial reproductivo (Sinclair *y cols.,* 2016).

Las modificaciones en la expresión de genes que no son resultado de una alteración en el DNA, pero que son heredables, se denominan modificaciones/cambios epigenéticos. Las células en tejidos adultos tienen la capacidad de mantener una huella epigenética del desarrollo embrionario (Hon *y cols.,* 2013) o incluso de generaciones pasadas. Por lo tanto, el estudio de los efectos de las tecnologías reproductivas en el patrón epigenético de los embriones y la descendencia resultante ha ganado más atención en los últimos años.

El modelo de producción bovino tanto de carne como de leche dificulta la evaluación de los resultados adversos en la salud adulta de la progenie y, por lo general, solo se obtiene información de los efectos clínicamente observados en animales jóvenes. La industria bovina no acostumbra a dar parte de problemas en la salud perinatal y adulta de la descendencia (Blondin 2016), aunque aún se observa un mayor peso de los terneros al nacimiento cuando provienen de embriones PIV (Bonilla *y cols.,* 2014). Además, los terneros nacidos de embriones PIV usando semen sexado inverso, son más pesados en

comparación a los terneros nacidos después de la IA, mientras que los terneros nacidos de embriones PIV con semen convencional tienen un peso de nacimiento intermedio (Siqueira *y cols.,* 2017).

La función de un animal adulto depende no solo del entorno al que estuvo expuesto después del nacimiento sino también de las condiciones del ambiente al que estuvo expuesto durante el desarrollo embrionario, desde la fecundación hasta las primeras diferenciaciones en el estadío de blastocisto (Fleming *y cols.,* 2015).

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2 | OBJETIVOS DE LA TESIS

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El objetivo general de esta tesis es desarrollar un sistema de cultivo para mejorar la calidad y la viabilidad de embriones bovinos producidos *in vitro*. Con este propósito, los objetivos específicos del presente trabajo fueron los siguientes:

1- Analizar, a corto y a largo plazo, los efectos que provoca la privación de proteína en cultivo individual durante la formación del blastocisto bovino *in vitro*. A tal fin, se evaluaron parámetros de desarrollo y viabilidad embrionaria, parámetros de gestación y fenotipos de los individuos nacidos (Capítulo 1).

2- Estudiar cómo la falta de proteína en cultivo, los estadíos embrionarios, el sexo del embrión, el contenido de lípidos y el proceso de vitrificación influyen en los fenotipos de embriones y terneros (Capítulo 2).

3- Establecer si una concentración muy reducida de suero fetal bovino (0.1%) en cultivo desde el día 1 hasta el día 6 puede mejorar los porcentajes de blastocistos tempranos, sin perjuicio de la calidad del embrión, determinada como supervivencia a la criopreservación, contenido de lípidos, apoptosis y viabilidad de la gestación de los embriones producidos (Capítulo 3).

4- Cuantificar el HDGF total presente en el fluido uterino mediante *Multiple Reaction Monitoring* (MRM), e identificar los efectos de HDGF recombinante en el desarrollo de estadíos embrionarios específicos de

embriones cultivados *in vitro*. Y evaluar la viabilidad de la gestación con los embriones producidos y los fenotipos de los terneros al parto. También se analizó el dimorfismo en la expresión de HDGF en células endometriales cocultivadas con un embrión macho o un embrión hembra (Capítulo 4).

3 | CAPÍTULO I

Short- and long-term outcomes of the absence of protein during bovine blastocyst formation *in vitro*

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Reproduction, Fertility and Development. 2017, 29(6), 1064-1073

This work was supported by the Spanish Ministry of Economy and Competitiveness – MINECO-project AGL2012–37772 and AGL2012–39652, Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013–2017 (GRUPIN 14–114) and FEDER and by the European Union Seventh Framework Program (FP7/2007–2013) under the grant agreement n8311776. M. M. was supported by MICINN-RYC08–03454. A. M. was supported by Secretaría Nacional de Educación Superior, Ciencia, Tecnología e Innovación – Ecuador – II Fase 2013. The authors are members of the COST Action FA1201 Epiconcept: Epigenetics and Periconception environment.

Abstract

In cattle, individual *in vitro* embryo culture after Day 6 benefits development, allowing non-invasive analysis of culture medium. However, undefined supplements in culture reduce analytical reliability. In this study we assayed the short- and long-term performance of embryos after bovine serum albumin removal over a 24-h period in individual culture.

The absence of protein decreased embryo development and cell counts in the inner cell mass without affecting blastocyst sex ratio. However, the absence of protein produced embryos with an improved tendency to survive vitrification after 24 h in culture (P=0.07). After transfer to recipients, birth rates of embryos that had been cultured with protein tended to decrease (P<0.06) mostly as a result of a higher number of miscarriages (P<0.013), reflecting lower viability. Birthweight, gestation length, height and thorax circumference did not differ between embryos cultured with or without protein. In fresh blastocysts cultured without protein, gene expression analysis showed higher abundance (P,0.05) of insulin-like growth factor 2 receptor (*IGF2R*; imprinting) and activating transcription factor 4 (*ATF4*) and DNA-damage inducible transcript 3 (*DDIT3*; endoplasmic reticulum stress) transcripts, with DNA methyltransferase 3A (*DNMT3A*; imprinting) tending to increase (P=0.062). However, in hatched blastocysts that survived cryopreservation, glucose-6-phosphate dehydrogenase (*G6PD*) was overexpressed in embryos cultured without protein (P<0.01). The absence of protein results in fewer blastocysts but improved long-term viability after cryopreservation.

3.1. Introduction

Within the culture of most cell types, bovine serum albumin (BSA) is not essential but it frequently improves performance (Francis 2010). In embryo technologies, BSA has been a classical alternative to serum and co-cultures (Duque *et al.* 2003), improving survival of cryopreservation with intact (Gómez *et al.* 2008*a*) and biopsied embryos (Yotsushima *et al.* 2004). Beneficial effects are observed preferentially in long-term culture, where the absence of protein is clearly detrimental (Krisher *et al.* 1999; Duque *et al.* 2003; Orsi and Leese 2004). BSA preparations are widely used in cell cultures of different species and obviously in bovine embryo culture. The embryo incorporates exogenous protein from the culture medium (CM) by endocytosis in order to maintain the intracellular amino-acid pool (Thompson *et al.* 1998). However, BSA is not a defined protein compound because it carries lipids, amino acids, hormones, peptides, metals and other undefined low-molecular-weight molecules that determine a significant batch-to-batch variation (reviewed by Francis 2010).

In replacing albumin, probably the most used macromolecular supplementation is the non-metabolisable substitute polyvinyl-alcohol (PVA). In cattle, PVA has been used as a unique macromolecule during *in vitro* maturation (IVM) and *in vitro* culture (IVC; Duque *et al.* 2003; Gómez *et al.* 2003, 2004; Orsi and Leese 2004; Lim *et al.* 2007), resulting in both pregnancies and births (Hidalgo *et al.* 2003, 2005; Lim *et al.* 2007).

In cattle, entire *in vitro* development in simple medium and individual culture negatively affects developmental competence, embryo quality and survival of cryopreservation, with few authors reporting improvements of blastocyst development in long-term individual culture compared with group culture

(Goovaerts *et al.* 2010). To overcome negative effects of entire individual culture we used group-cultured embryos from Day-1 to Day-6 and a shorter *in vitro* individual culture step of 24 h (Muñoz *et al.* 2014*a*) starting from Day-6 morulae (90%) and early blastocysts. The CM used in our predictive work was synthetic oviduct fluid with amino acids and myo-inositol, modified with 6 g L^{-1} bovine serum albumin (SOFaaci-BSA; Rodríguez *et al.* 2007). Removal of BSA from SOFaaci results in a chemically defined embryo culture medium. Defined conditions will help in avoiding sanitary concerns, in normalising culture conditions and in facilitating reliable analysis of media. In addition, BSA withdrawal will enhance the commercial interest of non-invasive analytical techniques in culture media, because repeatability will increase and inter-laboratory comparisons will become possible.

In this study we analysed the effects of protein removal on blastocyst development in individual culture with respect to survival of cryopreservation, differential cell counts (as a primary test of embryonic quality), pregnancy rate (embryonic viability), gestation length and weight and morphometry of individuals born (long-term embryonic quality). According to the observed results and the protein restriction hypothesis, we selected and analysed the expression of a panel of candidate genes related to metabolism, endoplasmic reticulum (ER), oxidative stress and imprinting mechanisms, factors which may underlie the observed effects. To our knowledge, no other work has analysed the same developmental period in individual culture and defined conditions.

3.2. Materials and Methods

All experimental procedures were sanctioned by the Animal Research Ethics Committee of SERIDA (Agreement 02/02/2012) in accordance with the European Community Directive 86/609/EC. Ovaries were collected from cows that were slaughtered in commercial abattoirs (Matadero de León, León, Spain, mostly for Holstein cows; Matadero de Tineo, Tineo, for Asturiana de los Valles (AV) cows) and transported to the laboratory in 9mg mL^{-1} NaCl solution with antibiotics. All reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated.

3.2.1. *In vitro* embryo production

Ovarian follicles (3–8mm in diameter) were aspirated and oocytes enclosed in a compact cumulus (≥3 cell layers) with evenly granulated cytoplasm were selected for *in vitro* maturation as described previously (Gómez *et al.* 2008*b*). Briefly, the cumulus–oocyte complexes (COCs) were washed in TCM-199-HEPES and matured in bicarbonate-buffered TCM 199 with FSH, LH, 17b-oestradiol and 10% fetal calf serum. Approximately 50 COCs were cultured in 500 mL maturation medium in four-well dishes at 39ºC in 5%CO_2 in air with high humidity for 22–24 h. After IVM, oocytes were subjected to *in vitro* fertilization (IVF; Day 0) with frozen–thawed, sex-sorted or non-sex-sorted spermatozoa from Holstein and AV bulls following described procedures (Trigal *et al.* 2012*a* and Gómez *et al.* 2008*b*, respectively). Briefly, semen samples from sex-sorted bulls were layered on a Percoll gradient (90% and 45%). After centrifugation the pellet was washed with Fert-TALP containing heparin and penicillamine, hypotaurine and epinephrine (PHE), centrifuged once more and the resultant

pellet was used for fertilisation. For non-sex-sorted spermatozoa, sperm separation was performed using the swim-up technique. Semen was layered down to a tube containing pre-equilibrated Sperm-TALP and the upper layer with motile spermatozoa collected after a 1-h incubation. The concentration of spermatozoa was determined with a haemocytometer. The COCs were washed and placed in four well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (non-sex-sorted spermatozoa) or with heparin+PHE (sex-sorted spermatozoa). *In vitro* fertilisation was accomplished by incubating oocytes and spermatozoa together for 18–20 h at 39ºC in 5% CO2 with high humidity. For experiments entirely *in vitro*, IVF was performed with non-sorted spermatozoa (*n*= 6 bulls), while embryos for transfer to recipients were produced with a combination of non-sorted and sex-sorted spermatozoa (*n*= 6 and *n*= 4 bulls, respectively). The embryo culture medium was synthetic oviduct fluid (SOF) containing essential and non-essential amino acids, citrate, myo-inositol and modified with or without 6mg mL^{-1} BSA. Polyvinyl alcohol (PVA; 0.5 mg mL^{-1}) was added as a BSA replacement. Presumptive zygotes were cultured in groups in 50-mL droplets under mineral oil; 1–2 mL per embryo; 25–35 embryos per drop; 5% CO_2, 5% O_2 in saturated humidity. Subsequently, Day-6 morulae (90% approximately) and early blastocysts (10% approximately) were picked up and individually transferred to individual culture drops (12 mL) for 48 h (Day-6 to Day-8; individual culture) of either SOF+6mgmL^{-1} BSA or SOF+0.5 mgmL^{-1} PVA. Media were renewed on Day-3 and Day-6. Blastocyst development, expansion and hatching rates were analysed on Day-7 and Day-8.

3.2.2. Differential cell counts

Cell counts were taken in Day-8 expanded blastocysts. Embryonic cells in the inner cell mass (ICM) and trophectoderm (TE) were differentially counted using a propidium iodide–bisbenzimide method (Trigal *et al.* 2012*a*). Briefly, blastocysts were incubated in BSA-free TCM-199-HEPES with 1% Triton X-100 and propidium iodide for 30 s. Samples were fixed in ethanol with bisbenzimide (Hoechst 33342), stored overnight at 4ºC and then transferred directly to a glycerol droplet on a glass microscope slide. Cell counts were made using digital images obtained with an inverted microscope equipped with an ultraviolet excitation filter at 330–385 nm and a barrier filter at 420 nm (Olympus Spain). The TE cells were identified by their red fluorescence, whereas ICM cells appeared blue.

3.2.3. Vitrification of embryos

Vitrification procedures have been previously described in detail (Trigal *et al.* 2012*a*). Briefly, Day-7 and Day-8 excellent and very good expanding to expanded *in vitro*-produced (IVP) blastocysts were vitrified in two steps using fibre plugs (Cryo-Logic vitrification method (CVM); Cryologic, Australia). Vitrification solutions contained ethylene glycol, dimethyl sulfoxide (DMSO) and sucrose as described (Vajta *et al.* 1998). Samples were vitrified by touching with the hook in a supercooled block placed in liquid nitrogen. Embryos were warmed in one step (Caamaño *et al.* 2015) by directly immersing the fibre plug end in 0.25M sucrose, where the embryo was kept for 5 min. Prior to transfer, vitrified–warmed embryos were allowed to re-expand for 1–2 h *in vitro*. Subsequently, re-expanded embryos were washed twice and loaded in straws.

3.2.4. Warming and survival of vitrified embryos

In vitro survival rates were analysed after warming and subsequent *in vitro* culture of Day-7 and Day-8 vitrified embryos in SOF + 10% FCS for 48 h.

3.2.5. Oestrus synchronisation of recipients, embryo transfer and pregnancy and birth monitoring

Detailed procedures have been previously described (Hidalgo *et al.* 2004). Briefly, Holstein and Asturiana de los Valles heifers were synchronised in oestrus by using an intravaginal progestagen device (PRID Alpha) for 10 days combined with a prostaglandin $F_2\alpha$ (PG) analogue (Dynolitic; Pfizer, Madrid, Spain) injected 48 h before progestagen removal. Normally, cryopreserved Day-7 embryos show higher pregnancy rates than older embryos (Hasler 2000) and this led us to set up an embryo transfer study using only Day-7 vitrified embryos. On Day 7, single re-expanded, vitrified and warmed blastocysts were non-surgically transferred to recipients in the cranial third of the uterine horn ipsilateral to the corpus luteum (CL) under epidural anaesthesia. Pregnancy was diagnosed on Day 40 and on Day 62; miscarriages and birth rates were monitored in recipients transferred once or twice. However, bodyweight (BW), height at withers (HW), thorax circumference (TC), gestation length (GL) and daily weight (BW/GL), were recorded at birth in recipients transferred up to four times.

3.2.6. Gene expression analysis: RNA extraction, reverse transcription and quantification of mRNA transcript abundance

For gene expression studies, Day-7 embryos were analysed both before and after vitrification. Pools of *n*=10 Day-7 expanded blastocysts were collected fresh and pools of *n*=6 vitrified blastocysts were collected after hatching at 24 h in postwarming culture. Poly(A) RNA was extracted using the Dynabeads mRNA DIRECT Micro Kit (Ambion; Thermo Fisher Scientific Inc., Oslo, Norway) following the manufacturer's instructions and with minor modifications (Bermejo-Álvarez *et al.* 2008). After 10 min incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and washed twice with Washing Buffer A and Washing Buffer B. Next, RNA was eluted with Tris-HCl. Immediately after extraction, the reverse transcription (RT) reaction was carried out following the manufacturer's instructions (Epicentre Technologies Corp., Madison, WI, USA) using poly(T) primers, random primers and Moloney Murine Leukemia Virus (MMLV) high performance reverse transcriptase enzyme in a total volume of 40 mL to prime the RT reaction and to produce cDNA. Tubes were heated to 70ºC for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. They were then incubated at 25ºC for 10 min to favour the annealing of random primers, followed by 37ºC 60 min to allow RT of RNA and finally 858C for 5 min to denature the enzyme. All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, verified 29 February 2016) to span exon–exon boundaries when possible.

All quantitative polymerase chain reactions (qPCR) were carried out in duplicate on the Rotorgene 6000 Real-Time Cycler TM (Corbett Research, Sydney, Australia) by adding a 2-mL aliquot of each sample to the PCR mix (GoTaq qPCR Master Mix; Promega Corporation, Madison, WI, USA) containing the

specific primers selected to amplify glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), solute carrier family 2 (facilitated glucose transporter) member 1 (*SCL2A1*, formerly *GLUT1*), glucose-6-phosphate dehydrogenase (*G6PD*), glutathione peroxidase 1 (*GPX1*), superoxide dismutase 2, mitochondrial (*SOD2*, formerly *MnSOD*), DNA methyltransferase 3A (*DNMT3A*), insulin-like growth factor 2 receptor (*IGF2R*), beclin 1, autophagy related (*BECN1*), transmembrane BAX inhibitor motif-containing 6 (*TMBIM6*), activating transcription factor 4 (*ATF4*) and DNA-damage-inducible transcript 3 (*DDIT3*). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 3.1. Cycling conditions were 94ºC for 3 min followed by 35 cycles of 94ºC for 15 s, 56ºC for 30 s, 72ºC for 10 s and 10 s of fluorescence acquisition. Each pair of primers was tested to achieve efficiencies close to 1 and then the comparative cycle threshold (DDCT) method was used to quantify expression levels as described by Schmittgen and Livak (2008). To avoid primer dimer artefacts, fluorescence was acquired in each cycle at a temperature higher than the melting temperature of primer dimers (specific for each product, 80–86ºC). Then the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background was determined for each sample. The DCT value was determined by subtracting the endogenous control (an average of H2A Histone Family, Member Z (*H2AZ*) and actin, beta (*ACTB*)) CT value for each sample from each gene CT value of the sample. Calculation of DDCT involved using the highest sample DCT value (i.e. the sample with the lowest target expression) as a constant to subtract from all other DCT sample values. Fold-

changes in the relative gene expression of the target were determined using the equation 2–DDCT.

Table 3.1. Details of primers used for qPCR

Entrez gene symbol	Gene name	Accession number	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product length (bp)
H2AFZ	H2A histone family, member Z	NM_174809	AGGACGACTAGCCATGGACGTGTG	CCACCACCAGCAATTGTAGCCTTG	209
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034.2	ACCCAGAAGACTGTGGATGG	ATGCCTGCTTCACCACCTTC	247
SCL2A1	Solute carrier family 2 (facilitated glucose transporter) member 1	NM_174602.2	CTGATCCTGGGTCGCTTCAT	ACGTACATGGGCACAAAACCA	68
G6PD	Glucose-6-phosphate dehydrogenase	NM_001244135.1	CGCTGGGACGGGGTGCCCTTCATC	CGCCAGGCCTCCCGCAGTTCATCA	347
GPX1	Glutathione peroxidase 1	NM_174076.3	GCAACCAGTTTGGGCATCA	CTCGCACTTTTCGAAGAGCATA	116
SOD2	Superoxide dismutase 2, mitochondrial (formerly MnSOD)	S67818.1	GCTTACAGATTGCTGCTTGT	AAGGTAATAAGCATGCTCCC	101
DNMT3A	DNA methyltransferase 3A	AY271299	CTGGTGCTGAAGGACTTGGGC	CAGAAGAAGGGGCGGTCATC	318
IGF2R	Insulin-like growth factor 2 receptor	NM_174352.2	GCTGCAGTGTGCCAAGTGAAAAAG	AGCCCCTCTGCCATTGTTACCT	201
BECN1	Beclin 1, autophagy related	NM_001033627.2	GATTCTGGACCGTGTCACCAT	TGTGGACATCATCCTGGCTGG	166
TMBIM6	Transmembrane BAX inhibitor motif-containing 6	NM_001076414.1	GCTTTGGCTGTTCACAGAGC	TACGGTGATGTGGTACGCTG	150
ATF4	Activating transcription factor 4	NM_001034342.2	CCGAGATGAGCTTTCTGAGC	AGCATCCTCCTTGCTGTTGT	223
DDIT3	DNA-damage-inducible transcript 3	NM_001078163.1	GTCACTGCCTTTCTCCTTCG	GGGAGGTGTGTGTGACCTCT	217

3.2.7. Embryo sexing by PCR

The results of the gene expression study provided possible evidence that protein removal altered the embryonic sex ratio. Accordingly, the embryonic sex was determined in expanded blastocysts produced with and without protein by a built-in control PCR technique based on amelogenin amplification (Trigal *et al.* 2012*b*). This method is particularly appropriate when a fully accurate embryonic sex diagnosis is necessary.

3.2.8. Statistical analysis

Data from embryo development, cell counts and survival of vitrification and pregnancy, calving and miscarriage rates were analysed using the Proc GLM module of SAS/STAT (Version 9.2; SAS Institute Inc., Cary, NC, USA). The models included the following fixed effects: culture system, replicate, bull, bull and recipient breeds, as well as year and season for pregnancy and calving

rates. Birthweight, gestation length and various body measurements (height and thorax circumference) also included embryonic sex as a fixed effect. Least-squares mean (LSM) and their erros (±s.e.m.) were estimated for each level of fixed effects with a significant F-value. The Ryan–Einot–Gabriel–Welsch Q-test was used to compare the raw means of the levels from the fixed effects. For gene expression, data were analysed using the Sigma Stat (Jandel Scientific, San Rafael, CA, USA) software package. Student's *t*-test was performed to study the differences in expression values between fresh embryos cultured with or without protein and between vitrified embryos cultured with or without protein.

3.3. Results

3.3.1. Embryo development

Under the culture conditions assayed, embryos cleaved at ~85% on Day 3 and produced 40–55% morulae on Day 6. *In vitro* development of bovine Day-6 embryos cultured individually with BSA led to blastocyst rates superior to those without BSA (Table 3.2). On Day 7, the presence of protein led to increased proportions of blastocysts ($P<0.0001$) and the expansion rates tended to increase ($P=0.054$). On Day 8, blastocysts and expansion rates were significantly higher with protein ($P<0.05$ and $P<0.01$, respectively) than without. Hatching rates *in vitro* were not affected at any time point. The presence of protein did not lead to obvious microscopic differences in blastocyst morphology.

Table 3.2. *In vitro* development of Day-6 embryos produced in group culture with SOFaaci with BSA (6 g L^{-1}) and subsequently cultured individually in 12-µL drops of SOFaaci with protein (6mg mL^{-1} BSA) or without protein (0.5mg mL^{-1} PVA) up to Day 8.

		Day 7 (%)		Day 8 (%)		
Protein	***n***	**Blastocysts**	**Expanded**	**Blastocysts**	**Expanded**	**Hatched**
(+)	618	71.3 ± 2.7^{x}	46.6 ± 3.1	80.6 ± 2.9^{a}	66.4 ± 3.3^{x}	7.1 ± 1.7
(-)	733	51.6 ± 2.7^{y}	38.4 ± 3.0	71.7 ± 2.8^{b}	54.3 ± 3.3^{y}	4.0 ± 1.7
P<		0.0001	0.054	0.043	0.0079	0.17

n = number of Day-6 embryos (cultured individually in 20 replicates).
Data are LSM of replicates ± s.e.m.
^{a,b}Values within columns with different superscript letters differ significantly P<0.05
^{x,y}Values within columns with different superscript letters differ significantly P<0.01

3.3.2. Differential cell counts

The absence of protein decreased cell counts in the ICM (*P*<0.05) and no other analysed variable was affected (i.e. TE, total cells and ICM/total cell rate; Table 3.3).

Table 3.3. Differential cell counts in Day-8 expanded blastocysts cultured individually from Day 6 to Day 8 in drops of SOFaaci with protein (6mg mL^{-1} BSA) or without protein (0.5 mg mL^{-1} PVA)

Protein	*n*	ICM	TE	Total	ICM/TOTAL
(+)	44	32.3 ± 1.2^{a}	106.9 ± 8.0	115.3 ± 4.4	23.6 ± 2.0
(-)	34	25.4 ± 1.7^{b}	102.3 ± 6.0	122.8 ± 4.9	20.7 ± 1.5

Data from four replicates. Data are LSM of replicates ± s.e.m.
ICM, inner cell mass; TE, trophectoderm.
^{a,b}Values within columns with different superscript letters differ significantly P<0.05

3.3.3. *In vitro* survival of vitrification and warming

After warming, a trend was observed in increased survival rates of vitrified embryos cultured in medium deprived of protein at 2 h and 24 h (*P*=0.09 and *P*=0.07, respectively; not shown in tables). In contrast, hatching rates at 24 h for Day-7 embryos cultured without protein were higher (*P*<0.03) than all Day-8 embryos produced with or without protein (Table 3.4). This was not observed with Day-7 embryos produced with BSA, which did not differ from their Day-8 counterparts. Differences between treatments observed with expansion and hatching at 2 h and 24 h were all suppressed 48 h after warming. Almost all

embryos (138/139) cultured without protein and vitrified on Day 7 re-expanded 2 h after warming.

Table 3.4. *In vitro* survival after vitrification and warming of Day-7 and Day-8 bovine expanded blastocysts that were cultured individually in synthetic oviduct fluid (SOF) medium with protein (6mg mL^{-1} BSA) or without protein (0.5 mg mL^{-1} PVA) from Day 6 onwards.

Protein as of	Vitrification		Re-expansion (%)		Hatching (%)	
Day-6	Day	*n*	2h	24h	24h	48h
(+)	7	144	95.0±1.9	93.6±2.2	35.0±6.3^{ab}	71.8±5.6
(-)	7	139	99.4±1.9	98.1±2.2	38.7±6.1^{a}	75.4±5.5
(+)	8	69	92.5±2.7	89.9±3.0	21.2±8.7^{bc}	58.2±7.8
(-)	8	57	94.6±2.8	94.6±3.1	13.4±8.9^{c}	65.0±8.0

Expanded blastocysts vitrified from 17 culture replicates and warmed in 10 replicates.
Data are LSM of replicates ± s.e.m.
^{a,b,c}Values within columns with different superscript letters differ significantly P<0.025.

3.3.4. Embryo transfer to recipients and birth

Embryos were transferred to recipients after vitrification and warming (Table 3.5). Birth rates tended to increase (*P*=0.059) without protein. Interestingly, no differences were observed in pregnancy rates on Days 40 and 62, but an increased miscarriage rate took place after Day 40 in embryos cultured with protein (*P*<0.02). The paternal side did not affect the long-term outcome of embryo transfer. Thus, in the group of embryos cultured with protein, miscarriages (*n* = 6) were represented among *n* = 6 different bulls (Table 3.6). In contrast, only *n*=1 miscarriage occurred within embryos cultured in protein-free medium. The absence of protein did not subsequently affect calving weight, gestation length, height, thorax circumference or fetal daily growth (Table 6). However, calves ≥50 kg were born from embryos cultured in medium with (*n*=1/15) and without

(*n*=3/12) protein (non-significant differences; *P*>0.54). Birthweights per donor breed (Asturiana, 42.8 ± 3.9; Holstein, 39.2 ± 3.5) were consistent with

published breed averages by artificial insemination (Asturiana, 40.5 ± 7.4, Gutiérrez *et al.* 2007; Holstein, 43.4 ± 4.3, Lazzari *et al.* 2002).

Table 3.5. Bodyweight (BW), calves with bodyweight larger than 50 kg (≥50 kg), height at withers (HW), thorax circumference (TC), gestation length (GL) and average fetal daily weight gain (DG) of IVP calves born after transfer of bovine vitrified–warmed Day-7 expanded blastocysts derived from Day-6 embryos cultured individually in 12µL of synthetic oviduct fluid medium with protein (6mg mL^{-1} BSA) or without protein (0.5mg mL^{-1} PVA)

Protein	*n*	BW (Kg)	*n* ≥ 50Kg (%)	HW (cm)	TC (cm)	GL (days)	DG (g/day)
(+)	15	41.4±2.5	1 (7)	75.2±2.7	78.0±1.9	280±1.5	148±9
(-)	12	45.6±3.7	3 (25)	81.2±3.9	79.4±2.8	283±2.2	150±13
P=		0.39	0.54	0.50	0.79	0.31	0.47

Data are LSM ± s.e.m. of average calf values.
Pregnancies were allowed to reach term naturally (no labour induction)

Table 3.6. Embryo transfers (*n*) performed per bull, with expression of Day-40 pregnancies and miscarriage attained.

Bull	Sex Sorted	Protein (+)			Protein (-)		
		ET (*n*)	Day-40	Miscarriage	ET (*n*)	Day-40	Miscarriage
A	No	5	2	1	9	6	0
B	No	2	1	1	6	4	1
C	Female	6	4	1			
C	Male	3	2	1			
F	No	3	1	in course	5	2	in course
G	Female	3	1	1			
H	Female	1	0		2	1	0
I	Female	5	3	1	4	2	0
I	Male	4	1	0			
J	No	9	4	0			
K	Male	3	2	0	1	0	
K	Female	3	1	0			
Total		**47**	**22**	**6**	**27**	**14**	**1**

3.3.5. Gene expression study

Gene expression was analysed in blastocysts before and after vitrification and warming. For fresh embryos analysed on Day 7 (Fig. 3.1*a*) cultured without protein, expression of the imprinting related gene *IGF2R* was higher ($P<0.03$) and *DNMT3A* tended to increase ($P=0.062$; not shown in figures). In addition,

the genes *ATF4* and *DDIT3*, markers of endoplasmic reticulum stress, showed higher abundance in embryos cultured in protein free medium (P<0.03). After vitrification and warming (Fig. 3.1*b*), hatched blastocysts showed no changes in any of the analysed genes with the exception of *G6PD*, the pentosephosphate pathway rate-limiting enzyme, which was significantly overexpressed in embryos cultured without protein (P<0.01).

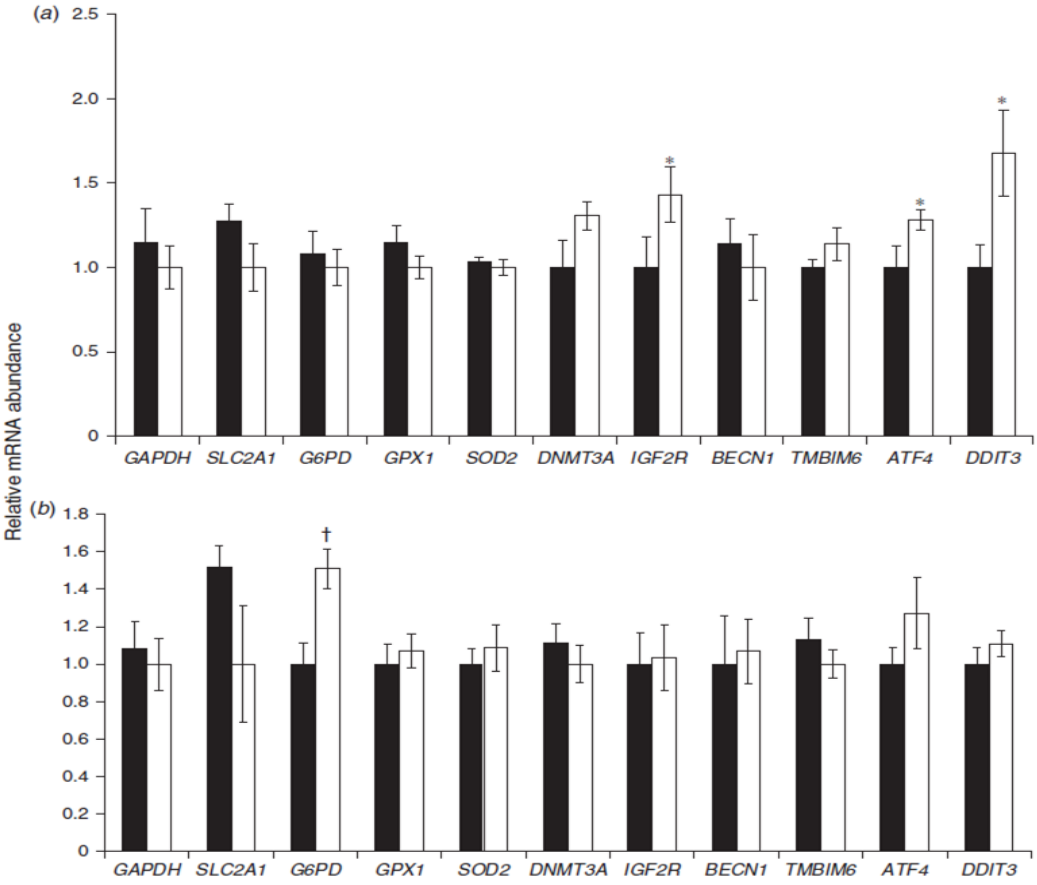

Fig. 3.1. Relative abundance (LSM ± s.e.m.; n=4 replicates) of mRNA transcribed from genes representing metabolism (*GAPDH*, *SLC2A1*, *G6PD*), oxidative stress (*GPX1*, *SOD2*), imprinting mechanisms (*DNMT3A*, *IGF2R*) and endoplasmic reticulum stress (*BECN1*, *TMB1B6*, *ATF4*, *DDIT3*). Analysis was performed on (*a*) pools of Day-7 fresh expanded blastocysts (n=10) and on (*b*) pools of hatched blastocysts (n=6) that survived 24 h *in vitro* after vitrification and warming of Day-7 expanded blastocysts derived from Day-6 embryos cultured individually in 12 µL of synthetic oviduct fluid medium with protein (6 mg mL^{-1} BSA; solid bars) or without protein (0.5mg mL^{-1} PVA; open bars). Superscripts express significant differences: *P<0.03, †P<0.01.

3.3.6. Sex analysis of Day-7 blastocysts

As *G6PD* can be overexpressed by female bovine embryos (Gutiérrez-Adán *et al.* 2000; Gutiérrez-Adán *et al.* 2004), we performed a sex determination study in expanded blastocysts. We did not find significant (*P>0*.14) differences in the male: female ratio (M: F) for embryos cultured with and without protein (M: F= 51 and M: F= 57, with *n*=50 and *n*=40 embryos sexed, respectively; not shown in tables).

3.4. Discussion

In this study we analysed the short- and long-term effects of the absence of protein during bovine blastocyst formation *in vitro*. Overall, the absence of protein during the morula-to-blastocyst transition yields fewer blastocysts but with improved developmental competence.

The absence of protein in culture is expected to impact embryonic protein and amino-acid stocks and metabolism. Interestingly, none of the classical carbohydrate sources provided for the embryo (glucose, lactate, pyruvate and citrate) was consumed by bovine *in vivo* embryos that were cultured *in vitro* or by embryos from pig, mouse and human (Krisher *et al.* 2015). In contrast, amino acids appear to be the main compounds consumed by embryos (Krisher *et al.* 2015; aspartate and serine consumed by cattle embryos).

In this study, the absence of protein from Day 6 reduced blastocyst and expansion rates on Days 7 and 8, as well as cell counts in the ICM. These effects are not surprising, as protein absence can negatively impact embryo development and cell counts in group embryo culture (Krisher *et al.* 1999; Duque *et al.* 2003; Orsi and Leese 2004). In cows, beneficial effects of protein are observed preferentially in long-term culture *in vitro*, whereby the absence of

protein is clearly detrimental, particularly in the second part of the culture (4 days; Krisher *et al.* 1999; Duque *et al.* 2003; Orsi and Leese 2004). Similar studies proposed that post-compaction stages would require more protein than earlier stages (Thompson *et al.* 1998), consistent with our current results and with previous work (Gómez *et al.*2014). After vitrification and warming, the absence of protein resulted in subtle benefits for Day-7 embryo survival *in vitro*. Since virtually all embryos were able to re-expand after 2 h of culture, this time-consuming and cost-increasing operation can now be suppressed for Day-7 embryos cultured for 24 h in protein-free medium. An important fact is that not one blastocyst hatched on Day 7 in culture without protein, while 5/144 embryos cultured with protein hatched (data not shown); this reduces sanitary constraints, as the use of embryos with shed zona pellucida is normally discouraged for cryopreservation and international exchanges (Stringfellow 2010). The reduction in the ICM cell count in embryos cultured without protein contrasts with the superior long-term viability shown by such embryos in the uterus. Thus, although it is generally assumed that minimum numbers of embryonic cells are necessary to establish pregnancy (Iwasaki *et al.* 1990), the optimal cell number and distribution of ICM and TE in the blastocyst are unclear. Higher cell counts in the ICM may lead to increased pregnancy rates (Loureiro *et al.* 2009). Conversely, embryos cultured in BSA-free medium showed no changes in cell counts and higher pregnancy rates than embryos cultured with BSA (Lim *et al.* 2007). In agreement with our findings, Lazzari *et al.* (2002) found that the *in vivo* environment (i.e. sheep oviduct) led to decreased cell counts as compared with *in vitro* culture in SOF with BSA or serum. We may conclude that the differences in birth and miscarriage rates

were not predicted from *in vitro* survival experiments. In our study, protein withdrawal led to reduced late miscarriage rates, suggesting that such a protein removal step is beneficial for pregnancy to term. In fact, cattle blastocysts living in the uterus trigger a decrease in both secreted uterine fluid volume, measured as total recoverable protein, and specifically albumin (Muñoz *et al.* 2012). As uterine passage is beneficial to embryonic viability and survival of cryopreservation (Rizos *et al.* 2002; Lonergan *et al.* 2004; Havlicek *et al.* 2010), the long-term beneficial effects we observed after a brief protein deprivation during late blastocyst development *in vitro* could be physiologically supported.

Although no effect was observed on birthweight, from a nutritional perspective, 24-h protein-free *in vitro* cultures may reflect, to a certain extent, early protein restriction in the uterine fluid of mother cows. In mice, blastocysts recovered from mothers fed with a low-protein diet during blastocyst development and transferred to non-restricted mothers result in heavier fetuses, showing that blastocyst growth can be programmed independent of the subsequent dietary environment (Watkins *et al.* 2008). In cattle, birthweight increased after the transfer of embryos cultured *in vitro* in SOF with serum or 16 g L^{-1} BSA (Lazzari *et al.* 2002), a concentration higher than that used in the present study. Such a study reported more than 50% calves 50 kg, in contrast with the lower proportion of large calves in our study (although more cases are necessary). Similar results were obtained by van Wagtendonck-de Leeuw *et al.* (2000) with an unknown BSA concentration. Dairy cows suffering negative energy balance (Leroy *et al.* 2015), which provides a metabolically altered embryonic environment, showed increased pregnancy losses during the first 60 days. In

contrast, our embryos with restricted protein showed limited presence (metabolic) or complete absence (oxidative) of genes expressing damage.

We found that the upregulation of *IGF2R* under protein removal resembles more physiological conditions than embryos cultured with protein. In contrast, downregulation of the imprinted gene *IGF2R* has been linked to fetal overgrowth (Young *et al.* 2001) and abortion in cattle clones (Yang *et al.*2013). In addition, dysregulation of specific paternal imprinting in the regulatory region of *IGF2R* (Bebbere *et al.* 2013) may explain the high incidence of placental failures in somatic cell nuclear transfer (SCNT) and IVP embryos (Heyman *et al.* 2002; Constant *et al.* 2006). Our embryos cultured with protein showed increased abortion rates, but no overweight fetuses were detected at birth. In fact, *in vivo*-developed conceptuses differ in the methylation status of *IGF2R* compared with IVP and SCNT conceptuses (Smith *et al.* 2015). These changes are not observed in individuals when born, regardless of their health status, which may explain the selective elimination of embryos carrying harmful epimutations before birth (Smith *et al.* 2015). During early development, stress sensitivity occurs at the time of nutritionally demanding processes, such as transcriptional activity during compaction, ion pumping required for blastocoel formation and lineage differentiation (Puscheck *et al.* 2015). Stressors such as nutrient starvation activate autophagy, a prosurvival process linked to endoplasmic reticulum (ER) stress that contributes to the recycling of old proteins and organelles.

In bovine embryos, inducing autophagy increases blastocyst development and trophectoderm cells and also reduces apoptosis, while autophagy inhibition counteracts these effects (Song *et al.* 2012). We hypothesised that recycling

proteins could be critical under protein restriction from Day-6 morula stage onwards, a time frame for stress sensitivity. The lack of differences in *GPX1* and *SOD2*, mitochondria-associated antioxidant enzyme-coding genes, indicates an absence of oxidative stress induced by protein withdrawal both before and after cryopreservation. In contrast, we found *ATF4* and *DDIT3* overexpressed in Day-7 blastocysts that were deprived of protein before cryopreservation, which confines the stress scope of protein restriction to the ER. Interestingly, these differences, together with the increased expression of *IGF2R* in fresh embryos, were not observed in hatched blastocysts that survived cryopreservation. In contrast, the absence of protein (Lim *et al.* 2007) and different systems of *in vitro* culture (with serum or BSA) did not alter the expression of *IGF2R* regarding *in vivo* development in the ovine oviduct or in the cow (Lazzari *et al.* 2002).

Suppressing ER stress appears to entail short-term beneficial effects on blastocyst development and quality (i.e. our protein group; Song *et al.* 2011, 2012; Yoon *et al.* 2014), while we have provided evidence of contrasting effects on pregnancy establishment and term development. Our results indicate that the absence of protein leads to ER stress beneficial for pregnancy maintenance and long-term viability.

Cryopreservation is a recognised stress source for embryos (Puscheck *et al.* 2015) so removal of stress markers after warming suggests that a certain grade of selection would take place. Interestingly, the different expression of *IGF2R* between groups also disappeared after warming and hatching *in vitro*.

This way, specific cohorts of embryos that hatch after warming would be able to progress through pregnancy to term, regardless of whether their culture

contained protein or not. Vitrification and warming suppressed differences in gene expression first observed in fresh embryos, leading to overexpression of *G6PD* in hatched blastocysts cultured without protein after warming. Activation of *G6PD* is indicative of response to oxidative stress and, in bovine blastocysts, may constitutively indicate dosage differences depending on embryonic sex (Gutiérrez-Adán *et al.* 2000; Kimura *et al.* 2004, 2005). Males and females differ in their resistance to heat-induced oxidative stress (Pérez-Crespo *et al.* 2005). In our study, nevertheless, no evidence of sex selection was observed during the morula-to-expanded blastocyst transition. Therefore, increases in *G6PD* should be associated with more viable and less miscarriage-producing embryos cultured in the absence of protein, perhaps reflecting a superior ability to counteract oxidative stress induced by cryopreservation. In cows and sheep increased *G6PD* activity is also observed in oocytes having lower lipid content (Castaneda *et al.* 2013; Mohammadi-Sangcheshmeh *et al.* 2014). Interestingly, fatty-acid carriage and uptake by albumin alters the fatty acid profile and enhances oxidative stress (Hughes *et al.* 2011), so culture without BSA could entail reduced lipid content in embryos, as shown in pigs (Romek *et al.* 2011). This investigation is in progress.

Many of the effects observed without protein are consistent with the quiet embryo hypothesis (Leese 2002; Leese *et al.* 2008), which suggests that viable embryos show low metabolic activity (including diminished amino-acid turnover and glycolysis). These ‘best’ embryos would respond better to stressors or would not need to respond to stressors. Removal of protein in culture may favour development of non-protein-dependent embryos (somewhat quiet), which could bear traits of improved viability such as higher survival of

cryopreservation. Protein absence on Day 6 and individual culture is potentially compatible with any other culture step before Day 6 given that individual culture provides blastocyst development equal to group culture and hatching rates that are superior to group culture (Muñoz *et al.*2014*b*). Even more interesting is the fact that, in the presence of protein, pregnancy rates with fresh IVP embryos were similar to fresh *in vivo* embryos cultured *in vitro* for 24 h (Muñoz *et al.* 2014*a*, 2014*b*). In the present study with vitrified embryos, the absence of protein led to a notable trend to increased birth rates by means of reducing miscarriage, which represents counteracting the cost-expensive and production damaging late-pregnancy losses in cattle farming. Interestingly, protein removal during blastocyst formation did not affect birthweight, gestation length or calf morphology. However, more studies are necessary to delineate these findings appropriately. Furthermore, individual culture provides rapid and direct access to CM as a substrate for non-invasive analysis, leading to biomarker discovery for prediction of pregnancy rates (Muñoz *et al.* 2014*a*, 2014*b*) or amino-acid turnover (Sturmey *et al.* 2010) and embryonic sex (Muñoz *et al.* 2014*c*). Users wishing to improve survival upon cryopreservation may allocate embryos to protein-free culture media in time with recipient availability or embryo surplus.

3.5. References

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4 | CAPÍTULO II

Protein in culture and endogenous lipid interact with embryonic stages *in vitro* to alter calf birthweight after embryo vitrification and warming

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Reproduction, Fertility and Development. 2017, 29(10), 1932-1943

Project support was provided by the Spanish Ministry of Economy and Competitiveness (MINECO; project AGL2012– 37772), Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013–2017 (GRUPIN 14–114) and FEDER. A. M. was supported by Secretaría Nacional de Educación Superior, Ciencia, Tecnología e Innovación, Ecuador, II Fase 2013. The authors are members of the COST Action FA1201 Epiconcept: Epigenetics and Periconception environment.

Abstract

Short-term protein removal *in vitro* improves long-term blastocyst competence to survive vitrification. We investigated the mechanisms and effects underlying protein removal. Day-6 morulae and early blastocysts were cultured individually with and without protein for 24 h. Development and lipid content were analysed in expanded blastocysts derived from morulae (M-XB) and from early blastocysts (EB-XB). Expression of genes involved in lipid metabolism, stress responses and apoptosis was analysed in fresh and vitrified–warmed M-XB produced with and without protein.

Pregnancy rates, birth rates and birthweight (BW) were recorded after transfer of embryos. Day-7 EB-XB production rates (with, 66.9 ± 6.2 and without, 68.8 ± 6.0 protein) were higher than M-XB rates (with, 21.4 ± 4.6 and without, 9.4 ± 4.6 protein; $P < 0.005$). EB-XB showed fewer lipids than M-XB ($P = 0.03$). In fresh M-XB, expression of sterol regulatory element binding protein (*SREBP1*) was lower with (4.1 ± 2.2) than without (13.6 ± 2.2) protein, contrary to results obtained for *Patatin-like phospholipase domain containing 2*, *Hormone-sensitive lipase* and *Bcl-2–associated X protein* ($P < 0.05$). Protein did not affect pregnancy rates and birth phenotypes ($P > 0.05$). However, BW was higher ($P < 0.01$) in calves born from vitrified M-XB (48.6 ± 3.4 kg) than from EB-XB (39.8 ± 2.9 kg). Such effects were more pronounced in females ($P < 0.001$). Calves from fresh embryos did not show BW differences. These results indicate that embryonic kinetics and vitrification impact birth phenotypes, at least in females. Alterations might involve exogenous protein and mobilisation of lipid stocks.

4.1. Introduction

Early embryo development is a highly dynamic process that can be influenced by numerous factors to which the embryo may adapt. Such adaptive capacity has been termed plasticity and it can have short- and long-term consequences, affecting blastocyst development and quality, the course of pregnancy and progeny health (Watkins *et al.* 2008). The period before implantation is of particular interest, as it has been identified as a critical window of developmental sensitivity to environmental or nutritional stress (Fleming *et al.* 2004).

Recently, we have shown that recipients of vitrified–warmed embryos cultured without protein between Day 6 and Day 7 show increased birth rates and reduced late miscarriage rates (Murillo-Ríos *et al.* 2016). The absence of protein selects embryos with improved developmental competence once vitrified, and we hypothesised that changes in lipid storage and metabolism could accompany the effects of protein removal (Murillo-Ríos *et al.* 2016). In fact, the culture environment increases lipid content in bovine *in vitro*-produced (IVP) embryos (Crosier *et al.* 2001; Lonergan *et al.* 2003) up to levels higher than that found *in vivo* collected (IVC) embryos (Crosier *et al.* 2001; González-Serrano *et al.* 2013). Bovine IVP blastocysts can develop well in culture with fatty acids (FA; Al Darwich *et al.* 2010) or with products of FA oxidation (Gómez 1997; Gómez *et al.* 2002). However, lipid metabolism is altered within IVP embryos when compared with IVC embryos (González-Serrano *et al.* 2013; Al Darwich *et al.* 2014). An excess of intracellular lipid increases embryo sensitivity to oxidative stress, chilling and cryopreservation (Abe *et al.* 2002; Reis *et al.* 2003; Sudano *et al.* 2011), and altered survival after cryopreservation may alter

pregnancy rates, gestation length and birthweight (BW; Bonilla *et al.* 2014). As the embryo develops to the expanded blastocyst (XB) stage, embryonic cells show fewer lipid granules (Abe *et al.* 2002; Barceló-Fimbres and Seidel 2011; Sudano *et al.* 2016) at the same time that they require more protein (Thompson *et al.* 1998) and amino acids (Guerif *et al.* 2013). Endocytosed protein maintains the intracellular amino-acid pool (Thompson *et al.* 1998) and the embryo can readily use amino acids and lipids in the absence of carbohydrates that feed the energy metabolism (reviewed by Krisher *et al.* 2015). With the onset of morula compaction and during blastocyst formation, energy demands, measured as oxygen and nutrient consumption, increase dramatically mainly due to two processes: (1) protein synthesis necessary for the embryo to initiate net growth and (2) the Na^+K^+ ATPase transport system that pumps sodium ions into the trophectoderm (TE) to form the blastocoel (Leese *et al.* 2008). Contrary to the blastocyst, in the morula the ability to use oxygen is still limited, which may hinder lipid catabolism. Protein availability could therefore determine the different lipid mobilisation characteristics of the early blastocyst (EB) and morula (M). Therefore, XB that derive from either morula or early blastocyst stages may show differences in lipid metabolism and viability after cryopreservation.

In the cow, embryos that develop *in vitro* show deviant kinetics in comparison to *in vivo* developing embryos (Merton *et al.* 2003; Sugimura *et al.* 2012; Liang *et al.* 2015). A known form of altered kinetics of bovine embryos is slow cleavage, which reduces development, with higher proportions of chromosomal abnormality, although slow-developing embryos may not show reduced pregnancy rates (Lonergan *et al.* 1999; Sugimura *et al.* 2012). Nevertheless, within embryos cleaving slowly, the incidence of abnormal direct passage from

one cell to 3–4 cells is higher than within fast-cleaving embryos (Sugimura *et al.* 2012). Altered passage also results in a higher incidence of chromosomal abnormality but lower pregnancy rates (Sugimura *et al.* 2010, 2012). Development patterns have not been thoroughly researched in the cow, despite the interest of studying developmental kinetics and its influence on neonatal phenotypes (Sugimura *et al.* 2012). In cattle, Merton *et al.* (2003) found that pregnancy rates increased when selecting Day-7 embryos for transfer as being derived from 'correct' Day-6 stage embryos. It could be that superior viability embryos are those that develop according to specific patterns (Gutiérrez-Adán *et al.* 2015) rather than embryos at specific embryonic stages. Kinetic studies in bovine embryos have usually analysed different stage endpoints, but not a same developmental endpoint reached by embryos that progressed from different earlier stages. The *in utero* development of IVP embryos bearing different development patterns to the XB stage is also unknown. Different embryonic development patterns can also be favoured by or adapted to changing progesterone (P4) concentrations, as early embryo development is dependent on the rate and timing of the P4 rise (Mann and Lamming 2001; Kenyon *et al.* 2013).

In this work we hypothesised that protein removal from *in vitro* culture would affect lipid content and underlying gene expression mechanisms differently depending on the embryonic stage affected. Therefore, we studied how specific embryonic stages develop under protein removal in terms of lipid content, embryonic sex ratio, embryo vitrification and calf phenotypes at birth.

4.2. Materials and Methods

All experimental procedures were approved by the Animal Research Ethics Committee of SERIDA (Agreement 02/02/2012), in accordance with the European Community Directive 86/609/EC. Ovaries were collected from cows that were slaughtered in commercial abattoirs (Matadero de Leon and Matadero de Guarnizo, mostly for Holstein ovaries and Matadero de Tineo for Asturiana de los Valles ovaries). Ovaries were transported to the laboratory in 9mg mL^{-1} NaCl solution with100 IU mL^{-1} penicillin and 100 mg mL^{-1} streptomycin sulphate and maintained at 25–30ºC. All reagents were purchased from Sigma unless otherwise stated.

4.2.1. Embryo production

In vitro-produced embryos were obtained as described (Trigal *et al.* 2012*a*) with minor modifications. Antral follicles (3–8mm in diameter) were aspirated through an 18-g needle connected to a syringe. Aspirated fluid was expelled into dishes containing holding medium (HM): TCM199 (Invitrogen), 25mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N0-(2-ethanesulfonic acid (HEPES) and 0.4 g L^{-1} bovine serum albumin (BSA) and a stereomicroscope was used to search for oocytes. Only good-quality oocytes with more than three layers of compact cumulus cells with homogenous cytoplasms were selected. For *in vitro* maturation (IVM), the cumulus–oocyte complexes (COCs) were rinsed three times in HM. Selected COCs were washed three times in maturation medium (MM) consisting of 2.2 g L^{-1} NaHCO3 in TCM199 supplemented with 10%(v/v) fetal calf serum(FCS), 1.5 mg mL^{-1} porcine FSH–LH (Stimufol; ULg FMV) and 1 mg mL^{-1} 17 β-oestradiol. COCs

were transferred (*n*=30–50) into a four well dish containing 500 mL of MM medium for 22–24 h at 38.7ºC in an atmosphere of 5% CO_2 with saturated humidity. After IVM, oocytes were subjected to *in vitro* fertilization (IVF; Day 0) with sex-sorted or non-sex-sorted spermatozoa following described procedures (Gómez *et al.* 2008; Trigal *et al.* 2012*a*). IVF was performed with commercial frozen–thawed spermatozoa from Holstein or Asturiana de los Valles breeds.

For experiments entirely *in vitro*, IVF was performed with non-sorted spermatozoa (*n*=6 bulls), while embryos for transfer to recipients were produced with non-sorted and sex-sorted spermatozoa (*n*=6 and *n*=4 bulls respectively). COCs were washed twice in HM and placed in four-well culture dishes containing pre-equilibrated fertilisation medium (Fert-Tyrode's albumin lactate pyruvate, TALP) with 10 mgmL^{-1} heparin (Calbiochem). IVF was accomplished by incubating oocytes and sperm cells together for 18–20 h at 38.7ºC in an atmosphere of 5% CO_2 with saturated humidity. Subsequently, cumulus cells were detached using a vortex and presumptive zygotes were cultured in glucose-free, modified synthetic oviduct fluid (mSOF) containing 45 µL mL^{-1} amino acids (BME Amino Acid Solution; Sigma) and 5 µL mL^{-1} non-essential amino acid (Minimum Essential Medium Non-Essential Amino Acid Solution; Sigma), 0.34mM tri-sodium-citrate, 2.77mM myo-inositol and 6 g L^{-1} BSA (Fraction V, mouse embryo culture tested A-3311) as previously described (Murillo-Ríos *et al.* 2016). Droplets of mSOF (1–2 mL per embryo) were layered under mineral oil and embryos cultured in groups of 35 to 45. *In vitro* culture was carried out at 38.7ºC, 5% CO_2, 5% O_2, 90% N_2 and saturated humidity. On Day 6 (143 h post-insemination (PI)), good-quality M (*n*=858) and EB (*n*=86) were selected and cultured individually for 24 h in 12-mL drops of mSOF with

(6mg mL^{-1} BSA) or without protein (0.5mg mL^{-1} polyvinyl-alcohol (PVA) was added to replace BSA) under mineral oil. Amino-acid supplements BME and MEM were used throughout. Blastocyst development was monitored on Day 7 (168 h PI and Day 8 (187 h PI).

4.2.2. Embryo sexing by PCR

We analysed embryonic sex as a factor influencing blastocyst development *in vitro*. The embryonic sex was determined in individually cultured Day-7 XB derived from Day-6 M (M-XB) and Day-6 EB (EB-XB), cultured with and without protein. A built-in control PCR technique based on amelogenin gene amplification (Trigal *et al.* 2012*b*) was used. Sex ratio was calculated as a proportion of male to female embryos.

4.2.3. Vitrification of embryos for transfer

Vitrification procedures have been previously described in detail (Trigal *et al.* 2012*a*). Briefly, Day-7 excellent XB were vitrified in two steps with fibre plugs (CryoLogic Vitrification Method; CVM). Final vitrification solutions contained 16.5% ethylene glycol, 16.5% dimethyl sulfoxide (DMSO) and 0.5M sucrose. Samples were vitrified by touching the surface of a chilled block placed in liquid nitrogen with the hook. Embryos were warmed by directly immersing the fibre plug end in sucrose solution in one step (Caamaño *et al.* 2015) or in two steps (Trigal *et al.* 2012*a*). Subsequently, embryos were washed twice in embryo preservation medium (IMV Technologies) and loaded into straws for transfer. *In vitro* development of vitrified–warmed embryos cultured with and without protein has already been reported (Murillo-Ríos *et al.* 2016).

4.2.4. Lipid and cell nuclei staining

Embryos selected were Day-6 morulae and Day-7 excellent- to very-good-quality blastocysts cultured with or without protein that derived from either morulae or early blastocysts. Embryos were fixed in 4% paraformaldehyde for 20 min at room temperature and kept in 0.1Mphosphate-buffered saline (PBS) with 0.1 mg mL^{-1} polyvinyl alcohol (PBS–PVA; pH 7.4, 4ºC) overnight. Nile red stock solution was prepared by dissolving 1mg mL^{-1} lipid-specific Nile red dye in DMSO, which was stored in the dark. Embryos were washed for 1 min with 400 mL of 1 mg mL^{-1} Nile red stock solution in PBS–PVA (based on Ghanem *et al.* 2014) and then stained for 1 h in darkness. Embryos were washed again in PBS–PVA and transferred into a 4well dish with 400 mL 4, 6-diamidino-2-phenylindole (DAPI) dye (5 mg mL^{-1} in PBS, 300nM concentration) for 2 min and washed thrice in PBS–PVA. Blastocysts stained only with DAPI were used as negative controls. Finally, embryos were mounted on a slide with Vectashield H-1000 (Vector Laboratories) under a coverslip. Samples stored in the dark at 48C overnight and examined under a confocal microscope (ultra-spectral Leica TCS-SP2-AOBS; Leica Microsystems). The lipophilic fluorescent Nile red dye was excited at 450–500 nm and DAPI at 421–476 nm. Next, digital pictures of the complete embryo, each 1.5 mm on the Z plane, were captured. Imaris 7.1 software (Bitplane Oxford Instruments) was used to generate three dimensional (3D) reconstructions from confocal datasets to assess lipid droplet contents. Lipid droplets (n) were measured in volume mode but arranged by diameter as small (<2 µm), medium (2–6 µm) and large (>6 µm), in order to facilitate comparisons with measurements in diameter from previous work (Abe et al. 2002).

4.2.5. RNA analysis

The expression of genes involved in lipid metabolism was analysed in fresh and vitrified–warmed embryos cultured with and without protein from Day 6 to Day 7 using previously described procedures (Cordova *et al.* 2014). All embryos used were Day-7 M-XB. Blastocysts were collected from single culture droplets, washed three times in PBS–PVA, snap frozen and kept at -80ºC until use. Total RNA was extracted from groups of blastocysts by using TRIzol reagent (Life Technologies). In brief, 300 µL of TRIzol was added to each tube with frozen embryos and 1 pg of luciferase RNA per embryo was added to each sample as external RNA control before extraction. Total RNA was then extracted according to the manufacturer's instructions. The total amount of RNA from each sample was subjected to reverse transcription (RT) using iScript cDNA Synthesis Kit (BioRad). Real-time polymerase chain reaction (PCR) was carried out in 20 mL containing 1 x qPCR Mastermix Plus for SYBR Green I (BioRad), specific primers (see Table 4.1) at a final concentration of 150nM and 5 µL of the RT reactions diluted 1 : 20. Real-time PCR quantification of gene expression was performed using a MyiQ thermocycler (BioRad). RNA extraction efficiency was checked by amplification of Luciferase transcripts and the samples with the lowest expression were removed from the analyses. Primer efficiency and the standard curve for each candidate gene were deduced from serial dilutions of the corresponding cDNA fragment as a template. The geometric mean of housekeeping genes (RPL19 and RPS9) was used to normalise gene expression. The relative amounts of gene transcripts (R) were calculated as R = gene $E^{-Ct\ gene}$/geometric mean (RPS9 $E^{-Ct\ RPS9}$; RPL19 $E^{-Ct\ RPL19}$), where E is PCR efficiency for each pair of primers (Table 4.1) and Ct is a cycle threshold.

In total, fresh XB (n=41 with protein and n=39 without protein) and vitrified–warmed hatched blastocysts (n=26 with protein and n=27 without protein) were analysed. Normalised mean values of gene expression were analysed separately for protein and no protein within the fresh and vitrified–warmed groups.

Table 4.1. Oligonucleotides used for real-time PCR gene expression 1 analysis

Gene	primer	Sequence 5'-3'	Accession	Amplicon (bp)	Gene product	Primers' efficiency (%)
ABHD6	fw	ACCCCGAAGGAGATGAGTGA	NM_001075196	276	Abhydrolase domain containing 6	1.93
	rev	CTGGGAGTTGGCGATTGACT				
ACACA	fw	TGCTTCCCATTTGCCATC	NM_174224	188	Acetyl coenzyme A carboxylase	1.90
	rev	CTGCCATCCTCACGACCT				
ACTB	fw	GCTGTCCCTGTATGCCTCTGG	NM_173979	349	Actin, beta	2.01
	rev	GAACCGCTCATTGCCGATGG				
BAX	fw	AGAGGATGATCGCAGCTGTGGA	NM_173894	300	Bcl-2–associated X protein	1.99
	rev	CAAAGATGGTCACTGTCTGCCATGT				
CPT1A	fw	TCCTGGTGGGCTACCAATTA	FJ415874	181	Carnitine palmitoyltransferase 1A	1.95
	rev	TGCGTCTGTAAAGCAGGATG				
CPT2	fw	TGTGCCTTCCTTCCTGTCTTGG	NM_001045889	111	Carnitine palmitoyltransferase 2	1.98
	rev	CGATGGGGTCTGGGTAAACGA				
DGAT1	fw	CGCCTTCTTCCACGAGTACC	NM_174693	159	Diacylglycerol O-acyltransferase 1	1.96
	rev	CCGATGATGAGTGACAGCCA				
FABP3	fw	ATCGTGACGCTGGATGGCGG	NM_174313	210	Fatty acid binding protein 3	2.04
	rev	GCCGAGTCCAGGAGTAGCCCA				
FABP5	fw	TGGCGCATTGGTTCAACATCAGG	NM_174315	193	Fatty acid binding protein 5	2.03
	rev	TGAACTGAGCTTGTTCATCCTCGC				
FASN	fw	CACTCCATCCTCGCTCTCC	AY343889	181	Fatty acid synthetase	2.03
	rev	GCCTGTCATCATCTGTCACC				
GLUT1	fw	CTGATCCTGGGTCGCTTCAT	NM_174602	68	Solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1)	2.04
	rev	ACGTACATGGGCACAAAACCA				
GPX1	fw	GCAACCAGTTTGGGCATCA	NM_174076	116	Glutathione peroxidase 1	2.06
	rev	CTCGCACTTTTCGAAGAGCATA				
GPX4	fw	CGATACGCCGAGTGTGGTTTAC	NM_174770	261	Glutathione peroxidase 4	1.96
	rev	ACAGCCGTTCTTGTCAATGAGG				
LIPE	fw	GAGTTTGAGCGGATCATTCA	NM_001080220	102	Hormone-sensitive lipase	1.98

	rev	TGAGGCCATGTTTGCTAGAG				
PLIN2	fw	ACAACACACCCCTCAACTGG	NM_173980	211	Adipophilin (perilipin 2)	1.97
	rev	CTGCCTGCCTACTTCAGACC				
PNPLA2	fw	ATGGTGCCCTACACTCTGCC	NM_001046005	152	Patatin-like phospholipase domain containing 2	1.88
	rev	AGCTTCCTCTTGGCGCGTAT				
PTSG2	fw	AGGTGTATGTATGAGTGTAGGA	NM_174445	483	Prostaglandin G/H synthase 2	2.05
	rev	GTGCTGGGCAAAGAATGCAA				
RBP4	fw	TTCGACAAGGCTCGCTTCGCC	NM_001040475	427	Retinol binding protein 4	2.02
	rev	CCTGCCTCTGCCGCACGATT				
RPL19	fw	AATCGCCAATGCCAACTC	NM_001040516	156	Ribosomal protein L19	1.98
	rev	CCCTTTCGCTTACCTATACC				
RPS9	fw	GGAGACCCTTCGAGAAGTCC	BC148016	180	Ribosomal protein S9	2.05
	rev	GGGCATTACCTTCGAACAGA				
SDHA	fw	GCAGAACCTGATGCTTTGTG	NM_174178	185	Succinate dehydrogenase complex, subunit A,‡	1.95
	rev	CGTAGGAGAGCGTGTGCTT				
SHC1	fw	GCAGTTGGAACCGGTAGCTT	NM_00116406	119	Src homology 2 domain containing) transforming protein 1 (p66Shc)	1.85
	rev	CCTTTGGTATAAGTGAGACCCG				
SREBP1	fw	ACCGCTCTTCCATCAATGAC	AB355703	190	Sterol regulatory element binding transcription protein 1	1.87
	rev	TTCAGCGATTTGCTTTTGTG				
TP53	fw	AGCTGGTGTTGGTAGGCAGT	NM_174201	180	Tumor protein p53	2.01
	rev	CCTCACCATCATCACACTGG				
YWHAZ	fw	GCATCCCACAGACTATTTCC	NM_174814	120	Tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein, zeta	2.05
	rev	GCAAAGACAATGACAGACCA				

2.4.6. Embryo transfer, pregnancy diagnosis and calf phenotyping

Detailed procedures have been described (Hidalgo et al. 2004). Briefly, Holstein and Asturiana de los Valles recipient heifers (*n* = 63) were synchronised in oestrus by using an intra-vaginal progestagen device (PRID Alpha; Ceva Salud Animal) for 10 days combined with a prostaglandin F2α analogue (Dynolitic, Pfizer,) injected 48 h before progestagen removal. Prior to transfer, Day-7 XB that had been vitrified at 170–172 h PI were warmed and allowed to re-expand for 1–2 h *in vitro*. On Day 7 (225 ± 1.5 h after progestagen removal; fixed time),

blastocysts were non-surgically transferred to recipients in the cranial third of the uterine horn ipsilateral to the corpus luteum under epidural anaesthesia. Pregnancy was diagnosed on Day 40 and on Day 62; miscarriages and birth rates were monitored in recipients transferred once or twice. However, bodyweight (BW), height at withers (HW), thorax circumference (TC), gestation length (GL) and daily weight (BW/GL) were recorded at birth in recipients transferred up to four times. Plasma P4 was measured on Day 0 and Day 7 (before embryo transfer) in blood samples taken up into ethylenediamine tetraacetic acid (EDTA) vacuum tubes via coccygeal vein puncture. An enzyme-linked immunosorbent assay (ELISA) test operating on a 0–40 ng mL^{-1} scale (DRG Diagnostics) was used. The test was sensitive starting from 0.5 ng mL^{-1} and cross-reactivity from steroids other than P4 was less than 1%. Intra and interassay coefficients of variation were 6% and 7% respectively.

2.4.7. Experimental design

4.2.7.1. Experiment 1: Day-7 embryo development patterns and sex ratio after individual culture in the presence or absence of protein during 24 h Blastocyst development was monitored *in vitro* from morulae (M) and early blastocysts (EB) cultured from Day 6 onwards in individual droplets with and without protein. On Day 7, embryonic sex was determined in expanded blastocysts (XB) derived from Day-6M (M-XB) and Day-6 EB (EB-XB), cultured with and without protein, in order to establish if development patterns influenced embryonic sex ratio.

4.2.7.2. Experiment 2: analysis of gene expression after individual culture in the presence or absence of protein

Expression of genes involved in lipid metabolism, oxidative stress and apoptosis was analysed in fresh and vitrified–warmed embryos. Because we could not generate a sufficient number of EB-XB blastocysts for this analysis, only M-XB blastocysts were used. Two separate analyses were performed with and without vitrification.

4.2.7.3. Experiment 3: long-term effects of protein removal and development patterns

In vitro embryo production and cryopreservation influence pregnancy rates and calf phenotypes. In this study, we analysed pregnancy rates on Days 40 and 62, birth rates, miscarriage rates from Day 40 onwards and calving weight and other body measurements at birth after transfer of vitrified–warmed embryos on the basis of the presence of protein and embryonic stage on Day 6. Experiments included both a proportion of re-examined retrospective data (Murillo-Ríos *et al.* 2016) and new data. Ultimately, in order to demonstrate that pregnancy, birth and calf phenotypes were attributable only to embryo vitrification, we retrospectively analysed an additional dataset of fresh versus vitrified embryo transfers of Day-7 M-XB and EB-XB developed with protein. In those years (2010–2011), the vitrification procedure required multi-step warming; some different bulls were used and no embryo transfers without protein were performed. Therefore, such data were not connected for statistical purposes and are shown separately.

4.2.8 Statistical analysis

Data requiring normalisation were arcsine transformed and analysed using the Proc GLM module of SAS/STAT (Version 9.2; SAS Institute Inc.). In

experiments concerning embryo development, gene expression analysis and lipid granule evaluation, the fixed effects included were presence of protein, embryonic stage on Day 6 (not for gene expression) and replicate. For embryo transfer, pregnancy and birth rates, body measurements and birthweight, the effects included were embryonic stage on Day 6, presence of protein, bull, sex-sorted spermatozoa, recipient breed, year and season. Plasma P4 was analysed using year, season and recipient breed, as well as corpus luteum side (Trigal *et al.* 2014), as fixed effects. Calf body measurements and birthweight also included embryonic sex. Least-squares means and their errors (± s.e.m.) were estimated for each level of fixed effects with a significant F-value. The Ryan–Einot–Gabriel–Welsch Q-test was used to compare the raw means of the levels from the fixed effects ($P<0.05$).

4.3. Results

4.3.1. Embryo development in individual culture

This experiment consisted of n=944 Day-6 embryos cultured in 20 replicates. On Day 7, blastocyst formation and expansion rates were higher for Day-6 EB than M, both with and without protein (P<0.005; Table 4.2). Within M, those developed with protein reached total blastocyst rates higher than those in protein free medium (74.5 ± 2.8 vs. 55.4 ± 2.5 respectively; P<0.005). On Day 8, similar to Day 7, higher expansion rates occurred in EB than in M (P<0.005). Interestingly, hatching rates were significantly increased (P<0.05) in EB cultured with protein, with the remaining groups showing lower and comparable hatching rates.

Table 4.2. *In vitro* blastocyst development rates of Day-6 morulae (M) and early blastocysts (EB) cultured individually in 12 µL drops of SOF containing amino-acids, citrate and myo-inositol with (+; 6mg mL^{-1} BSA) or without (-; 0.5 mg mL^{-1} PVA replacing BSA) protein up to Day 8

			Day-7 blastocysts (%)		Day-8 blastocysts (%)		
Protein	Stage	*n*	Total	Expanded	Total	Expanded	Hatched
(+)	EB	40	98.8±3.8^{a}(39)	66.9±6.2^{a}(28)	92.8±5.6^{a}(36)	86.6±7.4^{a}(35)	19.9±4.6^{a}(9)
(+)	M	400	74.5±2.8^{b}(296)	21.4±4.6^{b}(83)	77.5±4.1(311)	50.1±5.4^{bc}(202)	3.0±3.3^{b}(11)
(-)	EB	46	95.1±3.6^{a}(44)	68.8±6.0^{a}(30)	92.2±5.4^{a}(42)	68.8±7.1^{ab}(32)	5.9±4.4^{b}(3)
(-)	M	458	55.4±2.5^{c}(255)	9.4±4.6^{b}(44)	64.3±4.1^{b}(292)	35.0±5.4^{c}(161)	2.9±3.3^{b}(13)
P value			0.005	0.005	0.005	0.005	0.05

n = Day-6 embryos (cultured in 20 replicates). Data expressed as frequency percentages ± s.e.m. of Day-6 cultured embryos that reached at least the indicated blastocyst stage (n embryos in parentheses). Values in the same column with different superscript letters differ significantly at the indicated levels.

4.3.2. Embryonic sex ratio

Embryonic sex was analysed in Day-7 XB (*n* = 120 embryos from six replicates). There were no significant differences in the embryonic sex ratio between M-XB and EB-XB cultured with or without protein (P > 0.05; Table 4.3). However, there were significantly more female embryos among EB-XB than among M-XB (sex ratio: 32.0% vs. 52.6% respectively; P = 0.031).

Table 4.3. Expanded blastocyst sex ratio (percent males) according to their Day-6 origin from the morula or the early blastocyst stages and subsequent individual culture in 12 µL drops of SOF with (+; 6 mg mL^{-1} BSA) or without (-; 0.5 mg mL^{-1} PVA replacing BSA) protein.

Day-6 stage	Protein to Day-7	% Males (*n* males/total)
Morula	(+)	49.1 (27/55)
Early blastocyst	(+)	33.3 (5/15)
Morula	(-)	57.5 (23/40)
Early blastocyst	(-)	30.0 (3/10)
Cumulative		
Morula		52.6 (50/95)[a]
Early blastocyst		32.0 (8/25)[b]

[a,b] Values with different superscripts differ significantly (P<0.031).

4.3.3. Lipid content study

Lipid droplets were analysed in Day-6 morulae and M-XB and EB-XB cultured with or without protein (total 61 embryos; n = 7 biological replicates). On a per-cell basis, M showed a lipid granule load significantly higher than XB (P<0.001), regardless of embryos having been cultured in the presence or absence of protein (Fig. 4.1). Subsequently, the effects of protein and embryonic stages on lipid content and cell count were determined in Day-7 M-XB and EB-XB (*n* = 44 embryos; *n*=4 biological replicates). EB-XB contained more cells than M-XB (113.5 ± 3.8 and 102.7 ± 3.4 respectively; *P* = 0.03). However, cell numbers were similar in the presence and absence of protein in culture (109.3±3.9 and 106.5±3.4 respectively). The embryonic stage on Day 6 interacted with the

protein in affecting total lipid content (P = 0.03; Fig. 4.2). Thus, M-XB cultured without protein tended to contain fewer lipids than M-XB cultured with protein (P=0.053), whereas the EB-EX lipid droplets were similar in the presence or absence of protein. When lipid granules were arranged by size (Fig. 4.3), embryonic cells in M-XB cultured with protein showed more droplets <2 µm than M-XB cultured without protein and EB-XB cultured with protein (P<0.03), while EB-XB cultured without protein did not differ. Protein showed a significant effect (P = 0.02), but embryonic stage did not show an effect. The other sizes of granules (i.e. 2–6 µm and >6 µm), measured on a stage protein basis, did not differ and are not shown in figures. However, granules 2–6 µm tended to be more abundant in M-XB (22.2 ± 1.5) than EB-XB (17.4 ± 1.7; P = 0.057). Fig. 4.4 shows a 3D reconstruction from the confocal datasets that illustrate the lipid droplet distribution (Fig. 4.4a–b) and their size (Fig. 4.4c–e).

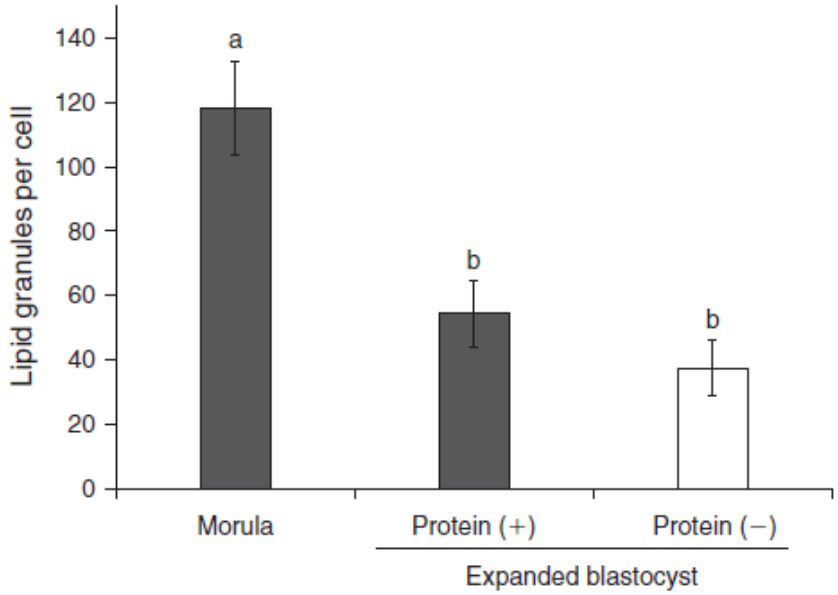

Fig. 4.1. Lipid content per embryonic cell in Day-6 morulae and Day-7 expanded blastocysts cultured with or without protein from Day 6 to Day 7. [a,b]Different superscript letters denote significant differences (*P*<0.001).

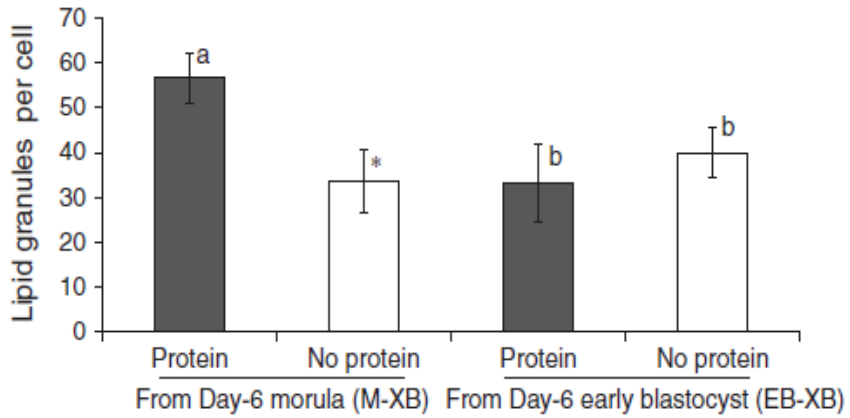

Fig. 4.2. Lipid granules per embryonic cell counted in Day-7 expanded bovine blastocysts following single culture of Day-6 morulae (M-XB) or early blastocysts (EB-XB) in SOF with protein (solid bars; 6mg mL^{-1} BSA) or without protein (open bars; 0.5 mg mL^{-1} PVA). [a,b]Different superscript letters denote significant differences (P<0.05) or a tendency (a,*: P<0.053).

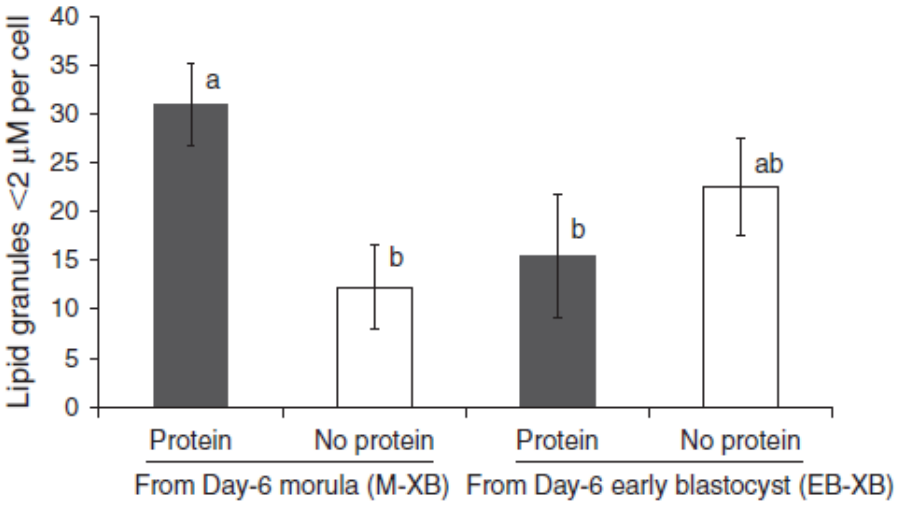

Fig. 4.3. Average cell abundance of lipid droplets <2 µm in diameter in Day-7 expanded bovine blastocysts following single culture of Day-6 morulae (M-XB) or early blastocysts (EB-XB) in SOF with protein (solid bars; 6 mg mL^{-1} BSA) or without protein (open bars; 0.5 mg mL^{-1} PVA). [a,b] Different superscript letters denote significant differences (P<0.03).

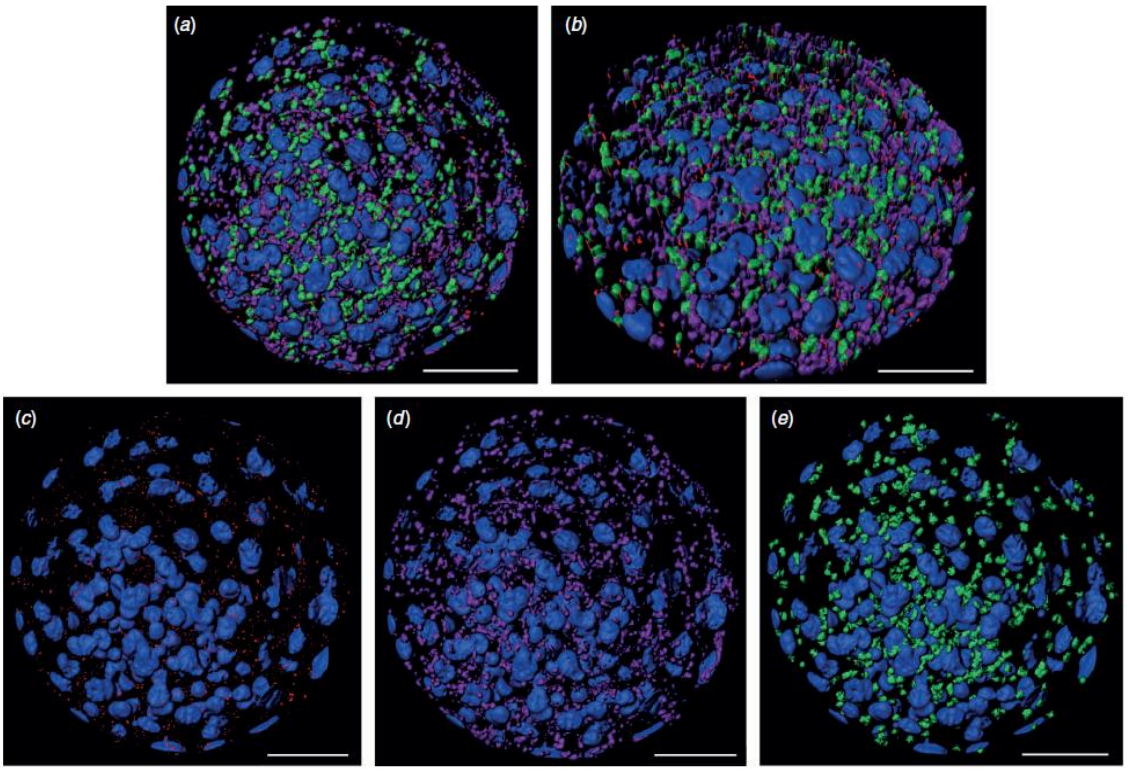

Fig. 4.4. Representative three-dimensional reconstructions of confocal datasets showing lipid droplets stained with Nile red in a Day-7 bovine expanded blastocyst. Lipid droplets have been pseudocoloured according to their size: <2 µm (red), 2–6 µm (purple), >6 µm (green). Lipid droplets are visualised concurrently (*a*, frontal view; *b*, oblique view) or (*c*–*e*) partitioned by size. Nuclei were counterstained with DAPI (blue). Scale bars = 50 µm.

4.3.4. Gene expression study

The effects of the presence of protein from Day 6 to Day 7 of *in vitro* embryo development on lipid metabolism-related gene expression were analysed in Day-7 M-XB before and after vitrification and warming (as hatched embryos). Studies with fresh XB consisted of *n* = 83 embryos and six replicates and studies with vitrified–warmed hatched embryos consisted of *n* = 53 embryos in five replicates. We compared the relative expression of genes involved in FA synthesis (*acetyl coenzyme A carboxylase* (*ACACA*) and *fatty acid synthetase* (*FASN*), lipid storage (*adipophilin* (*perilipin 2*) (*PLIN2*) and *diacylglycerol O-acyltransferase* (*DGAT*)), FA transport (*fatty acid binding protein 3* (*FABP3*) and *fatty acid binding protein 5* (*FABP5*)), lipolysis (lipases *hormone-sensitive lipase*

(*LIPE*), *patatin-like phospholipase domain containing* (*PLPLA*), *ABHD6*) and mitochondrial b-oxidation (*CPT1A*, *CPT2*). Most of these genes were similarly expressed in Day 7 fresh embryos cultured with and without protein, as shown in Fig. 4.5. However, sterol regulatory element binding protein (*SREBP1*), a transcription factor regulating FA metabolism, showed reduced expression in the presence of protein ($P<0.05$). In contrast, the lipolysis-related genes *PLPLA* and *LIPE* were 2-fold more abundant in the presence of protein ($P<0.05$). Genes encoding glutathione peroxidases 1 and 4 did not differ, but *glutathione peroxidase 1(GPX1)* expression tended to increase ($P<0.08$) in fresh embryos cultured with protein (Fig. 6). No differences were detected in the expression of *solute carrier family 2* (*facilitated glucose transporter*)*, member 1* (*SLC2A1* or *GLUT1*; involved in glucose transport), *prostaglandin G/H synthase 2* (*PTGS2*; responsible for prostaglandin synthesis) or in the house-keeping genes *actin, beta* (*ACTB*), *tryptophan 5-monooxygenase activation protein, zeta* (*YWHAZ*) and *Succinate dehydrogenase complex, subunit A* (*SHDA*). Protein in culture triggered overexpression of the pro-apoptotic gene *Bcl-2–associated X protein* (*BAX*) (Fig. 4.6). After vitrification and warming, no significant differences were found in hatched blastocysts cultured with or without protein.

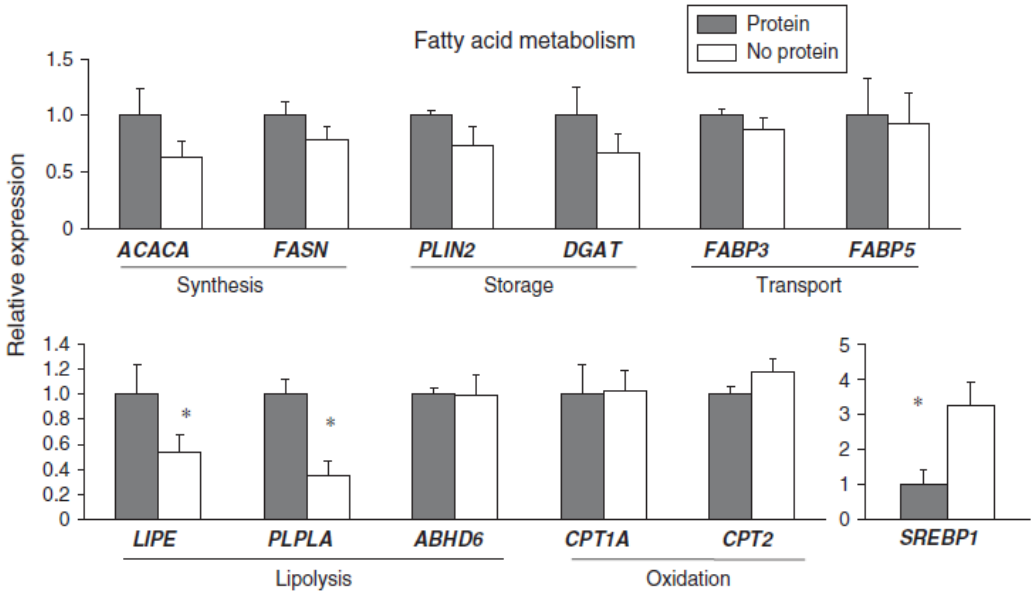

Fig. 4.5. Real-time PCR quantification of relative abundance of transcripts from genes involved in lipid fatty-acid metabolism in Day-7 fresh expanded blastocysts derived from Day-6 morulae (M-XB) cultured for 24 h with (solid bars) or without (open bars) protein. *Asterisks denote significant differences (P<0.05).

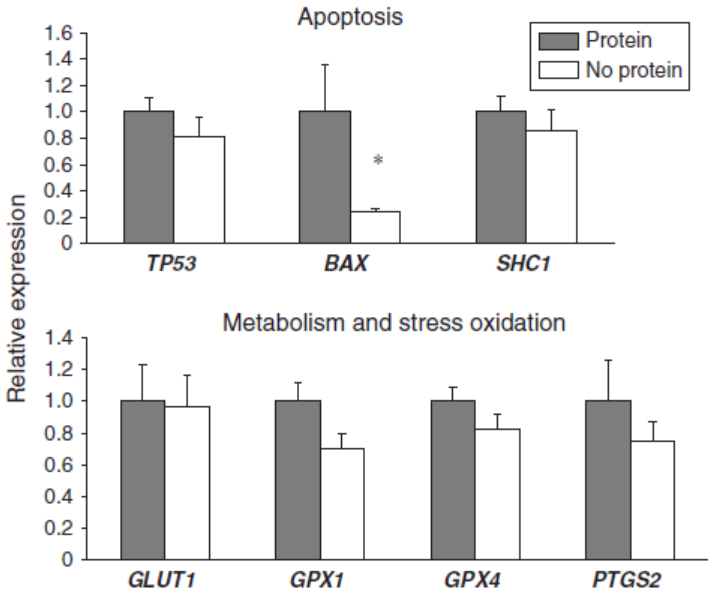

Fig. 4.6. Real-time PCR quantification of relative abundance of transcripts from genes involved in metabolism, lipid oxidative metabolism, antioxidative and stress defence and apoptosis in Day-7 fresh expanded blastocysts derived from Day-6 morulae (M-XB) cultured for 24 h with (solid bars) or without (open bars) protein. *Asterisks denote significant differences (P<0.05).

4.3.5. Embryo transfer, pregnancy, birth and calf phenotypes

Vitrified–warmed Day-7 M-XB and EB-XB, cultured with or without protein, were transferred to recipients (Table 4.4). Plasma P4 concentration increments did not change between pregnant and open recipients according to the embryonic development pattern (i.e. M-XB or EB-XB; Table 4.5) or protein in culture (Table 4.6).

Table 4.4. Pregnancy, calving and miscarriage rates after transfer of bovine vitrified Day-7 expanded blastocysts in terms of their Day-6 origin from the morula or the early blastocyst stage and subsequent individual culture in 12-µL drops of SOF with (+; 6 mg mL^{-1} BSA) or without (-; 0.5 mg mL^{-1} PVA replacing BSA) protein

Day-6 stage	Protein to Day 7	Pregnancy rate (%)		Birth	Miscarriage From Day-40 (%)
		Day 40	Day 60		
Morula	(+)	16/36 (44)	15/36 (42)	9/35 (26)	6/15 (40)
Blastocyst	(+)	7/12 (58)	7/12 (58)	6/12 (50)	1/7 (14)
Morula	(-)	14/22 (64)	13/22 (59)	11/20 (55)	2/13 (15)
Blastocyst	(-)	9/20 (45)	8/20 (40)	6/14 (43)	0/6 (0)

Pregnancy and birth rates expressed as % (n pregnant/n transferred at each specific endpoint; i.e. some pregnancies were in course). Miscarriage rates include premature stillbirth and are calculated only with those Day-40 pregnancies that did or should have reached term

Table 4.5. Progesterone increases from Day-0 up to Day-7 in recipients transferred with Day-7 expanded blastocysts in terms of their Day-6 origin from the morula or the early blastocyst stage and their pregnancy status on Day-40

Day-6 stage	Pregnant	*n*	P4 [Day-7] – [Day-0]
Early blastocyst	(+)	13	15.3±3.5
Early blastocyst	(-)	14	12.5±3.0
Morula	(+)	27	17.1±2.1
Morula	(-)	27	18.8±2.7

Differences were not significant (P>0.10)

Table 4.6. Progesterone increases from Day-0 up to Day-7 in recipients transferred with Day-7 expanded blastocysts in terms their culture with or without protein from Day-6 onwards and their pregnancy status on Day-40

Day-6 culture	Pregnant	*n*	P4 [Day-7] – [Day-0]
Protein	(+)	22	18.4±3.0
Protein	(-)	26	14.7±2.6
No protein	(+)	18	14.7±2.7
No protein	(-)	15	16.5±2.9

Differences were not significant (P>0.10)

Interaction between Day-6 stages and protein did not affect pregnancy or birth rates (P>0.05; Table 4.4). Miscarriage rates after Day 40 were not significantly affected (P>0.10), although M-XB cultured with protein registered numerically higher pregnancy losses. At birth, bodyweight, height at withers, thorax circumference, gestation length and average daily gain weight were not significantly affected by the interaction between stage and protein (P>0.05; data not shown). However, calves from M-XB showed significant increases in bodyweight and average daily gain weight (P>0.01) over calves that were EB-XB (48.6 ± 3.4 kg and 172 ± 11 g day^{-1} vs. 39.8 ± 2.9 kg and 140 ± 9 g day^{-1} respectively; Table 4.7).

Table 4.7. Weight and morphometry of calves born after transfer of bovine vitrified–warmed Day-7 expanded blastocysts in terms of their Day-6 origin from the morula or the early blastocyst stage

Day-6 Stage	*n*	BW (Kg)	HW (cm)	TC (cm)	GL (days)	DG (g day^{-1})
Blastocyst	12	39.8 ± 2.9^{a}	78.9 ± 3.5	80.6 ± 2.3	281.7 ± 2.3	140 ± 9^{a}
Morula	21	48.6 ± 3.4^{b}	83.0 ± 3.9	84.6 ± 2.6	281.2 ± 2.7	172 ± 11^{b}

Bodyweight (BW), height at withers (HW), thorax circumference (TC), gestation length (GL) and average daily weight gain (DG) of the fetus. Data are LSM ± s.e.m. ^{a,b}Values in the same column with different superscript letters differ significantly (P<0.01)

Table 4.8. Weight and morphometry of female calves born after transfer of bovine vitrified–warmed Day-7 expanded blastocysts according to their Day-6 origin from the morula or the early blastocyst stage

Day-6 stage	*n*	BW (Kg)	HW (cm)	TC (cm)	GL (days)	DG (g day^{-1})
Blastocyst	9	38.8 ± 2.2^{a}	72.9 ± 2.9	77.7 ± 1.9	284.4 ± 2.6	135 ± 7^{a}
Morula	17	49.5 ± 2.3^{b}	78.6 ± 2.9	82.3 ± 2.0	285.1 ± 2.6	173 ± 7^{b}

Bodyweight (BW), height at withers (HW), thorax circumference (TC), gestation length (GL) and average daily weight gain (DG) of the fetus. Data are LSM ± s.e.m. ^{a,b}Values in the same column with different superscript letters differ significantly (P<0.001)

When analysis included only female calves, M and EB stage effects on birthweight and average daily gain weight were strikingly more pronounced

(*P*<0.001; Table 4.8). Fig. 4.7 shows plotted birthweights per bull, sex and embryonic stage arising from vitrified embryos.

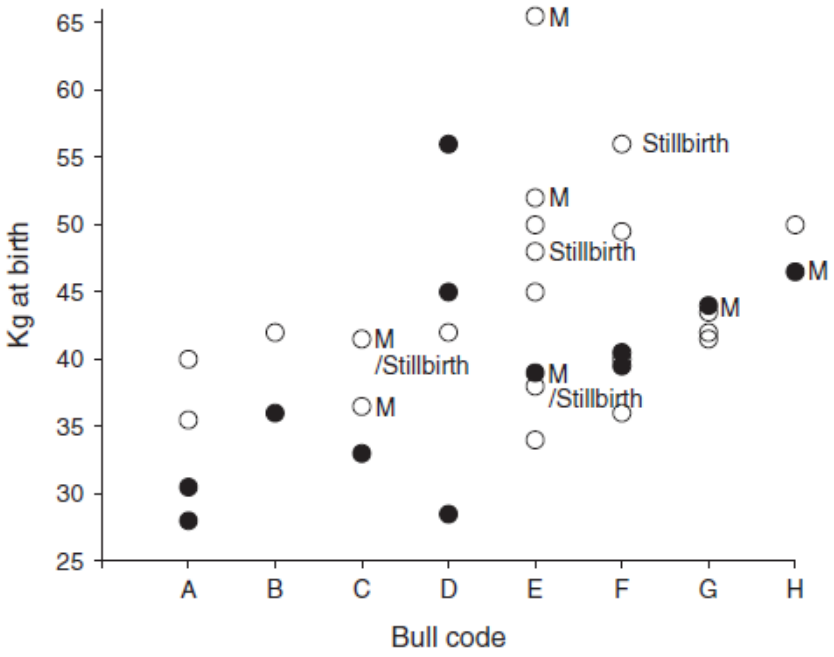

Fig. 4.7. Birthweight on a per bull (A to H) basis of calves born after transfer of Day-7 IVP vitrified–warmed embryos whose development stages on Day 6 were monitored as compact morulae (M-XB; open circles) or early blastocysts (EB-XB; solid circles). Males are shown as 'M'; the remainder are females.

Subsequently, we retrospectively analysed birth data of fresh versus vitrified–warmed embryos cultured only with protein (Table 4.9). Calves born from vitrified embryos showed a pronounced tendency to differ (*P*<0.06) in birthweight and average daily gain weight between M-XB and EB-XB (44.8 ± 2kg vs. 38.1 ± 2.4 kg, respectively). In contrast, fresh embryos did not show these tendencies (40.0 ± 2.3 kg vs. 43.2 ± 3.2 kg for M-XB and EB-XB respectively; *P*>0.05). Gestation was shorter in vitrified compared with fresh embryos (278.7 ± 2.1 days vs. 286.8 ± 2.3 days; *P*<0.03).

Table 4.9. Weight and morphometry of calves born after transfer of bovine fresh vs. vitrified/warmed Day-7 expanded blastocysts in terms of their Day-6 origin from the morula or the early blastocyst stage and subsequent individual culture in 12-µL of SOF containing protein (6 mg mL^{-1} BSA)

Day-6 stage	Day-7 vitrification	*n*	BW (Kg)	HW (cm)	TC (cm)	GL (days)	DG (g/day)
Blastocyst	Vitrified	5	38.1±2.4[a]	72.2±2.5	80.1±2.0	279.0±2.7	136±8[a]
Morula	Vitrified	11	44.8±2.0[b]	75.6±1.8	82.6±1.5	278.4±2.3	160±7[b]
Blastocyst	Fresh	6	43.2±3.2	73.1±3.7	84.1±2.9	287.2±3.7	151±11
Morula	Fresh	13	40.0±2.3	74.3±2.3	80.6±1.8	285.3±2.6	140±8

Bodyweight (BW), height at withers (HW), thorax circumference (TC), gestation length (GL) and average daily weight gain (DG) of the fetus. Calves born to recipients transferred one to four times. Data are LSM ± s.e.m. [a,b]Values in the same column with different superscript letters denote a tendency (P<0.06)

4.4. Discussion

We describe here how embryo development patterns in the presence or absence of protein entail short- and long-term effects after transfer of vitrified–warmed bovine embryos. In cattle *in vitro* embryo production, post-compaction stages require more protein (Thompson *et al.* 1998) and amino acids (Guerif *et al.* 2013) to develop than cleavage stages. In this study protein withdrawal reduced blastocysts rates from the Day-6 M stage but not from the EB stage. However, amino-acid metabolism of embryos that transit from the M to the EB stage is less active than in embryos that progress from the EB to the XB stage (Guerif *et al.* 2013). Therefore, the different effects of protein restriction on Day-6 stages could not be explained only on the basis of amino-acid shortage. Perhaps protein restriction affects compaction without interfering with later events such as cavitation and blastocyst expansion, since disruption of genes involved in compaction interferes with blastocyst formation (Barcroft *et al.* 2004). In this study we observed higher rates of female embryos within EB-XB compared with M-XB. In cattle, when no previous development pattern is considered, more males appear among the faster-developing blastocysts than

females (Gutiérrez-Adán *et al.* 1996; Larson *et al.* 2001; Kimura *et al.* 2005), although a 24-h individual embryo culture in mSOF with protein did not skew overall blastocyst sex ratio (Muñoz *et al.* 2014*a*, 2014*b*). Discrepancies could be explained by reduced proportions of EB among Day-6 embryos (≈10%), as sex ratio is influenced in only a minimum, unnoticed number of the embryos. Interestingly, the sex effects we observed were clearly stage-associated effects and protein did not exert any influence, in spite of male and female embryos differing in amino-acid metabolism (Sturmey *et al.* 2010) and in the regulation of protein in uterine fluid (Gómez *et al.* 2013). In addition, morulae and blastocysts differ in the numbers of expressed sex-linked genes and such differences increase as the embryo develops to the blastocyst stage (Denicol *et al.* 2015). Protein in culture changed lipid content in XB emerging from Day-6M and EB. Embryonic cells in M contain more lipid granules than in EBs (Sudano *et al.* 2016). Furthermore, protein removal increased the reduction in lipid content in the transition from the M to the XB stage, as shown in the pig blastocyst (Romek *et al.* 2011). However, lipid stocks were reduced in EB-XB, regardless of the presence or absence of protein. Therefore, both exogenous protein and Day-6 embryonic stage (i.e. M or EB) affected lipid content observed at the Day-7 XB stage. It must be considered that our protein supplement may contain FA and the albumin-bound FA fraction may actively incorporate FA into the embryonic cells (Hughes *et al.* 2011).

Expression patterns of lipid metabolism and oxidative stress response markedly contrast between blastocysts generated *in vivo* and *in vitro* (Rizos *et al.* 2002; González-Serrano *et al.* 2013; Sudano *et al.* 2014), which highlights the importance of lipid regulation for embryo quality. Within fresh blastocysts, the

changes in gene expression from the absence of protein were circumscribed to lipid breakdown and apoptosis. The sterol regulatory element binding protein (*SREBP1*) was overexpressed in fresh M-XB cultured without protein. SREBP1 activates genes encoding enzymes of cholesterol and FA biosynthesis when intracellular sterol levels are low. Typically, upregulation of *SREBP1* occurs in embryos cultured *in vitro* over *in vivo*-collected embryos (Driver *et al.* 2012; González-Serrano *et al.* 2013). However, free, exogenous and endogenous FA activates *SREBP1* in the blastocyst (Al Darwich *et al.* 2010; González-Serrano *et al.* 2013), by which increased free FA derived from lipid breakdown in embryos cultured without protein would activate *SREBP1*. The decrease in the lipolytic genes *PLPLA* and *LIPE* can also be explained as being subsequent to a lipid breakdown. In the cow, *LIPE* is expressed during mobilisation of body fat reserves (Elis *et al.* 2013). Differences in the size of lipid granules in bovine IVP embryos were previously described (Abe *et al.* 2002; Barceló-Fimbres and Seidel 2011). Lipogenesis is associated with the smallest granules, which converge to form larger ones by different mechanisms (Thiam *et al.* 2013). Thus, M-XB without protein showed fewer small and total lipid granules than M-XB with protein, suggesting that protein removal led to lipid breakdown. Consistent with our previous observations (Murillo-Ríos *et al.* 2016), *BAX* expression was reduced in embryos cultured without protein. Within bovine embryos, *BAX* overexpression is associated with lower-quality embryos (Vandaele *et al.* 2008). In any case, reduced lipid content, lipid mobilisation and improved survival after cryopreservation accompanied a low apoptotic index in bovine embryos (Ghanem *et al.* 2014).

Specific traits of gene expression shown by fresh embryos disappeared in hatched blastocysts that survived vitrification. We have previously observed that vitrification suppresses differences in gene expression after thawing (Murillo-Ríos *et al.* 2016). Excess of intracellular lipid increases embryo sensitivity to oxidative stress, chilling and cryopreservation (Abe *et al.* 2002; Reis *et al.* 2003; Sudano *et al.* 2011), by which lipid mobilisation would be a pre-requisite for fresh IVP bovine embryos to survive cryopreservation better (Sanches *et al.* 2013; Takahashi *et al.* 2013; Ghanem *et al.* 2014). Development patterns to the XB stage and vitrification–warming had long-term consequences. Differences in lipid stocks and lipid metabolism between M and EB might be the partially underlying cause of the traits we observed in vitrified embryos during in utero development. In this study, although differences in miscarriage rates were not significant, M-XB with protein had the highest lipid content and number of miscarriages, which is worthy of further research.

Birthweight was affected by embryo development patterns within vitrified–warmed embryos. Calves, particularly females, arising from M-XB were heavier and showed higher daily weight gain than EB-XB. Females were highly represented in our sample set because of the use of female sex-sorted spermatozoa for IVF. The variety of bulls used, with only one out of eight bulls showing discrepant results, and the fact that pregnancies were allowed to reach term naturally, without birth induction, probably reflects the effects of treatments on birth phenotypes better. Birthweight was not directly affected by protein restriction. *In vivo*, mammalian mothers fed low-energy and low-protein diets can compensate for this restriction in the uterine interface (Spiegler *et al.* 2014) with no birthweight effects. However, birthweight of rats and mice can be

affected by low-protein diets administered early in development (Fleming *et al.* 2015). Gestation length was longer for cows receiving a fresh embryo than for cows receiving a vitrified–warmed embryo, contrasting with the information given by Bonilla *et al.* (2014). However, in our work, vitrification only affected birthweight on a Day-6 embryonic stage basis, which contrasts with the work carried out by Bonilla *et al.* (2014), who reported a trend to increased birthweight in female calves from vitrified embryos. Our study indicated that EB showed lower substrate requirement than M for developing to the XB stage. The turnover in metabolite uptake and substrate production is lower in more competent embryos (Leese *et al.* 2008). The amino acid extra load represented by the protein in culture does not seem to be required by the EB-XB, which mobilised their lipids without protein deprivation. These traits favoured reduction in birthweight after vitrification. The link between embryonic stages, birthweight and vitrification was demonstrated with several bulls and both one-step and two-step vitrification, as well as the absence of effects in fresh embryos. Male calves can express birthweight changes within IVP embryos better (Camargo *et al.* 2010) and we obtained clear differences among female calves. However, caution is necessary, as the number of male calves obtained is not large enough to determine a possible sex effect on birthweight. Vitrified–warmed embryos show extended damage in inner cell mass (ICM) cells (Gómez *et al.* 2009). The birthweight of calves is positively correlated with placental weight and cotyledonary size and weight (Sullivan *et al.* 2009). In our study, cell damage in the ICM or trophectoderm (TE) of EB-XB and M-XB surviving vitrification was not assessed. Consequently, possible involvement of vitrification in placenta formation, capable of explaining birthweight differences,

remains unknown. The P4 increase measured did not vary with development patterns in vitrified embryos and pregnancy.

Improvements in IVP require also carrying pregnancies to term and exploring the health of the offspring. Short-term, individual culture offers biotechnological advantages. In the case of no recipients available or embryo surplus, EBs can be assigned to protein deprivation and vitrification with no overweight calves at birth. A disadvantage of our procedure is that only 10% of embryos reached the EB stage on Day 6 and the M to XB development rates are lower without protein. However, we have tripled the EB-XB rates without protein by adding minute proportions of serum from Day 1 to Day 6 (Mullaart *et al.* 2015), before protein deprivation.

In conclusion, embryonic kinetics during blastocyst expansion greatly affect short- and long-term viability, with contrasting effects on embryo development rates and phenotypes of calves born when the embryos are vitrified, at least in the female subset. Therefore, it is possible to manipulate calf phenotypes affecting essential parameters such as birthweight by introducing simple changes in culture conditions combined with selection of embryos under specific development patterns before vitrification.

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5 | CAPÍTULO III

Low serum concentration in bovine embryo culture enhances early blastocyst rates on Day-6 with quality traits in the expanded blastocyst stage similar to BSA-cultured embryos

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Reproductive Biology 2017. *17(2), 162-171*

Project support was provided by the Spanish Ministry of Economy and Competitiveness-MINECO-project AGL2016- 78597-R, Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013-2017 (GRUPIN 14-114) and FEDER. AM was supported by Secretaría Nacional de Educación Superior, Ciencia, Tecnología e Innovación – Ecuador – II Fase 2013. The authors are members of the COST Action FA1201 Epiconcept: Epigenetics and Periconception environment.

Abstract

In bovine, single *in vitro* embryo culture in protein-free medium from Day-6 to Day-7 leads to expanded blastocyst (XB) with improved pregnancy and birth rates after cryopreservation. Under these conditions, early blastocysts (EB) progress to the XB stage at higher rates than morulae (M). However, embryo production with BSA in culture prior to Day-6 leads to low EB rates. We investigated whether a very low FCS concentration (0.1%) in culture from Day-1 to Day-6 would improve EB rates and, subsequently, increase XB rates on Day-7 after single culture in protein-free medium. The quality of embryos produced was evaluated in terms of survival to cryopreservation, apoptosis percentage, lipid accumulation and transfer to recipients. On Day-6, EB rates from embryos cultured with FCS were higher than with BSA ($P = 0.022$). On Day-7, XB rates were higher in embryos from Day-6 EB than from Day–6 M, both with and without FCS ($P < 0.005$). After vitrification/warming of Day-7 XB, 100% embryos survived at 24 h in all treatments, and total cell number and apoptosis percentage were not affected by the presence of FCS or embryonic stage on Day-6. Cryopreserved and fresh embryos produced with FCS until Day-6, and then deprived of protein and cultured individually, led to pregnancies after ET. In conclusion, minute FCS concentration improves EB rates on Day-6 leading, after one-day single culture without protein, to more XBs. The quality of XB produced with FCS compares well with XB produced with BSA in terms of apoptosis, lipid accumulation and pregnancy.

5.1. Introduction

Defined *in vitro* culture media (CM) have been created to help avoiding sanitary concerns and to normalize culture conditions. In addition, defined CM improves repeatability, inter-laboratory comparisons, and facilitates reliable analysis of media [1–3]. In bovine, compared with group culture, long-term single culture in simple medium reduces embryo development rates, embryo quality, survival to cryopreservation and pregnancy viability [4]. Nevertheless, we observed that an individual culture limited to after Day-6 improved embryo development competence, allowing embryo traceability and non-invasive analysis of CM, such as prediction of pregnancy rates [5,6], and embryonic sex [7,8]. We have recently shown that single embryo culture for 24 h in a protein-free medium, resulted in Day-7 embryos with improved birth rates after vitrification and transfer to recipients [9]. Furthermore, protein removal did not affect birth weight, gestation length or morphometry of calves in comparison with embryos cultured with protein [10]. In previous studies, we observed that one-day single culture without protein from Day-6 onwards increased rates of Day-7 expanded blastocyst that derived from Day-6 early blastocysts (EB-XB) over those from Day-6 morulae (M-XB) [9,10]. Furthermore, calves born after transfer of vitrified/warmed embryos from Day-7 EB-XB have lower birthweight than calves derived from Day-7 M-XB [10]. In cattle, high birthweight increases the risks of dystocia, fetal death, neonatal mortality, and damage to the dam [11,12]. However, in our culture conditions with BSA prior to Day-6 only 10% of embryos reach the early blastocyst stage on Day-6. Thus, improving early blastocyst rates on Day-6 is necessary in order to obtain more expanded blastocysts on Day-7. *In vitro* produced bovine embryos at the expanded blastocyst stage on

Day-7 lead to the highest pregnancy rates both as fresh and cryopreserved [5,13–15]. Serum supplementation in embryo culture media at concentrations of 1% to 20% (v/v) has usually been used to enhance kinetics of embryo development and blastocyst rates [16–20]. However, with high serum concentrations in CM, *abnormalities* and deficiencies in embryo quality also have been described [19–25]. As a strategy to minimize detrimental effects of serum supplementation, serum concentrations in the CM were reduced as much as possible. Thus, Mullaart et al. [26] showed that a very low serum concentration (0.1%, v/v) throughout the entire culture improved blastocyst production. However, no data on embryo quality, survival of cryopreservation or pregnancy viability has been reported. Single culture without protein is potentially compatible with other previous culture steps [9,10]. We hypothesized that very low concentration of serum in culture, from Day-1 up to Day-6, could improve early blastocyst rates which, after single culture without protein, would subsequently increase expanded blastocyst rates on Day-7. However, adverse effects of serum on the embryonic quality [20,27] and viability [28] need to be evaluated, prior to introducing a large-scale, embryo transfer study. The objectives of this study were (1) to improve the expanded blastocysts production on Day-7 with the addition of 0.1% fetal calf serum (FCS) prior to a 24 h single culture without protein, and (2) to evaluate the quality of produced embryos in terms of survival to cryopreservation, apoptosis rates and lipid accumulation. In addition, a preliminary trial of embryo transfer (ET) was performed to evaluate the viability of fresh and cryopreserved embryos.

5.2. Material and Methods

All experimental procedures were approved by the Animal Research Ethics Committee of SERIDA (Agreement 02/02/2012), in accordance with the European Community Directive 86/609/EC. All reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated.

5.2.1. Oocyte collection and *in vitro* maturation (IVM)

Ovaries were collected from cows slaughtered in a commercial abattoir (Matadero de Guarnizo, Cantabria, Spain). Ovaries were transported to the laboratory in NaCl solution (9 mg/mL) with penicillin 100 IU/mL and streptomycin sulfate 100 mg/mL, and maintained at 25ºC to 30ºC. Antral follicles (3 to 8 mm in diameter) were aspirated through an 18-g needle connected to a syringe. Aspirated fluid was expelled into dishes containing holding medium (HM) TCM199 (Invitrogen, Barcelona, Spain), 25 mM HEPES and 0.4 mg mL^{-1} BSA. A search for oocytes was carried out using a stereomicroscope, and only good- quality oocytes with more than three layers of compact cumulus cells with homogenous cytoplasm were selected. For *in vitro* maturation (IVM), the cumulus–oocyte complexes (COCs) were rinsed three times in HM. Selected COCs were washed three times in maturation medium (MM) consisting of TCM199 $NaHCO_3$ (2.2 mg mL^{-1}) supplemented with 10% (v/v) FCS (SIGMA F4135), 1.5 mg mL^{-1} of porcine FSH-LH (Stimufol; ULg FMV, Liège, Belgium) and 1 mg mL^{-1} 17 β-estradiol. COCs were transferred (n = 30-50) along with 500 mL of IVM medium into each well of a four-well dish for 22–24 h at 38.7ºC in 5% CO_2 atmosphere with saturated humidity.

5.2.2. Sperm preparation and *in vitro* fertilization (IVF)

Commercial frozen sperm from an Asturiana de los Valles bull with proven fertility was thawed and used for IVF (Day 0). Motile sperm was obtained by a swim-up procedure [23]. Briefly, semen from one frozen straw corresponding to one bull was thawed in a water bath (30 s) and added to a polystyrene tube containing 1 mL of pre-equilibrated Sperm-TALP (Tyrode's albumin lactate pyruvate). After 1 h incubation, the supernatant upper layer containing motile sperm was recovered. Sperm was centrifuged for 7 min at 200 x g and the supernatant was aspirated to leave a pellet. The concentration of spermatozoa was determined with a hemocytometer. COCs were washed twice in HM and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert- TALP) with heparin (10 mg mL^{-1}; Calbiochem, La Jolla, CA, USA). Spermatozoa were added at a concentration of 2 x 10^6 cells mL^{-1} in 500 mL of medium per well, containing a maximum of 100 COCs. IVF was accomplished by incubating oocytes and sperm cells together for 18 to 20 h at 38.7 ºC in 5% CO_2 atmosphere with saturated humidity.

5.2.3. *In vitro* culture

Cumulus cells were detached using a vortex and fertilized oocytes were cultured in modified synthetic oviduct fluid (mSOF) containing amino acids (BME amino acids solution, 45 mL mL^{-1} and MEM non- essential amino acids solution, 3.3 mL mL^{-1}), citrate (0.1 mg mL^{-1}), myo-inositol (0.5 mg mL^{-1}), and BSA (6 mg mL^{-1}) with 0.1% (v/v) FCS (SIGMA F4135; n = 908) or without FCS (n = 1133). *In vitro* culture was carried out at 38.7 ºC, 5% CO_2, 5% O_2, 90% N_2, and saturated humidity. On Day-6 (143 h PI), excellent and good quality (grade

1 and grade 2 [29]) morulae and early blastocysts were selected and cultured individually in 12 mL mSOF with 0.5 mg mL^{-1} polyvinyl- alcohol PVA (instead of BSA) under mineral oil. Blastocyst development was monitored on Day-7 (168 h PI).

5.2.4. Embryo vitrification, warming and *in vitro* survival

Vitrification procedures have been described in detail [30]. Briefly, Day-7 excellent and good quality (grade 1 and grade 2 [29]) expanded blastocysts were vitrified in two-steps with fiber plugs (CryoLogic Vitrification Method; CVM). All procedures were performed in a warm room (25 ºC) on a heated surface (41 ºC). Embryos were first handled in a basic vitrification medium (VM), consisting of TCM 199-HEPES + 20% (v/v) FCS. Groups of one to five blastocysts were exposed to VM with 7.5% ethylene-glycol (EG), 7.5% DMSO (vitrification solution-1) for 3 min, and then moved into a microdrop containing VM with 16.5% EG, 16.5% DMSO and 0.5 M sucrose (vitrification solution-2; VS2). The time spent by the embryos in VS2 (including loading) was 20–25 s. Samples were vitrified by touching the surface of a supercooled block placed in LN_2 with a hook. Vitrified embryos were stored in closed straws for up 6 months in liquid nitrogen (-196 ºC) until warming. Embryos were warmed in one step [31] by directly immersing the fibreplug end in 800 mL of 0.25 M sucrose in VM, where the embryo was kept for 5 min and subsequently transferred and washed twice in VM. *In vitro* survival rates were analyzed after warming and subsequent *in vitro* culture of Day-7 vitrified embryos in droplets of 25 mL of mSOF containing 6 mg mL^{-1} BSA and 10% FCS (v/v; SIGMA F4135). Embryo survival was evaluated in terms of re-expansion and hatching rates at 24 and 48 h.

5.2.5. Apoptosis in blastocysts: TUNEL assay

Excellent and good quality hatched blastocysts (grade 1 and grade 2 [29]) surviving vitrification/warming were fixed in 4% paraformaldehyde with 0.1 mg mL^{-1} PVA and then washed and stored in phosphate buffered saline (SIGMA P4417) with 0.1 mg mL^{-1} PVA (PBS-PVA; pH = 7.4, 4 ºC) until use. Embryos were permeabilized for 40 min at room temperature with sodium citrate (0.1 M; pH = 6.0; SIGMA C8532) containing PVA (0.2 mg mL^{-1}) and Triton (0.1% v/v), and subsequently washed twice in PBS-PVA with Triton (0.5% v/v). Next, embryos were incubated on drops of 50 mL of Image iT-Tx Signal Enhancer (Invitrogen, I36933) for 30 min at room temperature, washed twice with PBS-PVA and incubated with labeling reagent according to the manufacturer's instructions (In situ Cell Death Detection Kit with Fluorescein, Roche1, Mannheim, BW, Germany). Positive controls for TUNEL were carried out by treating embryos with 10 IU mL^{-1} of DNase I (Takara, 2215A). All samples were incubated in micro-drops of the TUNEL Kit containing 10% of the enzymatic solution (deoxinucleotidil terminal transferase enzyme) with 90% of the labeling solution (20- deoxyuridine 50-triphosphate-dUTP + fluorescein isothiocyanate-conjugated-FITC) for 1 h in a humid chamber at 37 ºC in the dark. In a parallel assay, embryos were incubated in micro-drops containing only labeling solution as negative control. For nuclei counter- staining, embryos were incubated with 400 mL 4, 6-diamidino-2- phenylindole (DAPI) dye (300 nM in PBS) for 2 min at room temperature, washed in PBS-PVA solution, and finally mounted on a glass slide with Vectashield-H1000 (Vector Labs, USA) under a coverslip. Embryos were analyzed with a confocal microscope (ultra-spectral Leica TCS-SP8-AOBS; Leica Microsystems, Mannheim, Germany). An excitation wavelength of

488 nm was selected for detection of fluorescein-12-dUTP and a 405 nm wavelength to excite DAPI. Photomicrograph of serial optical sections were recorded every 1.5–2 mm vertical step along the Z-axis of each embryo. Total embryonic cells and DNA-fragmented nuclei were analyzed using software ImageJ (Confocal Uniovi ImageJ; version 1.51). Nuclei with green fluorescence (FITC) were considered TUNEL positive (fragmented DNA). Total healthy and apoptotic cells were assessed by DAPI staining of based on the presence of blue fluorescence. The results were expressed as total cells number and TUNEL-positive apoptosis percentage.

5.2.6. Lipids and cell nuclei staining and quantification

The following excellent and good quality embryos were selected to the analysis: (I) Day-6 morulae produced in the absence or the presence of 0.1% FCS, and (ll) Day-7 M-XB and EB-XB produced with or without 0.1% FCS up to Day-6, and subsequently individually cultured without protein for 24 h. Lipid dye and quantification procedures have been described in detail [10]. Briefly, embryos were fixed in 4% paraformaldehyde with 0.1 mg mL^{-1} PVA for 20 min at room temperature and kept in PBS-PVA overnight. Nile red (SIGMA N3013) stock solution was prepared by dissolving 1 mg mL^{-1} lipid-specific Nile red dye in DMSO, which was stored in the dark. Embryos were washed for 1 min with 400 mL of 1 mg mL^{-1} Nile red stock solution in PBS-PVA [39], and then stained for 1 h in darkness. Embryos were washed again in PBS-PVA, and transferred into a well dish with 400 mL DAPI dye (300 nM in PBS) for 2 min and washed three times in PBS-PVA. Blastocysts stained with only DAPI were used as negative control. Finally, embryos were mounted on a slide with Vectashield H-1000

(Vector Labs, USA) under a cover slip. Samples were stored in the dark at 4 ºC overnight and examined under a confocal microscope (ultra-spectral Leica TCS-SP8-X; Leica Microsystems, Mannheim, Germany). The lipophilic fluorescent Nile red dye was excited at 488 nm, which allows the observation of neutral lipids, including esterified cholesterol and triglycerides, and DAPI was excited at 405 nm. Next, digital photomicrographs of complete embryos, each 1.5 mm on Z plane, were captured using an immersion objective (HC-PL-APO-CS2 40x/1.30 oil) and acquisition software (Leica Application Suite X version 1.8.1). IMARIS 7.1.1 software (Bitplane Oxford Instruments, Zurich, Switzerland) was used to generate three-dimensional (3D) reconstructions from confocal datasets to assess lipid droplet contents. Only morphologically healthy nuclei stained with DAPI were counted. Lipid droplets (LD) were counted and measured in volume mode but arranged by diameter as small (<2 µm), medium (from 2 µm to 6 µm), and large (>6 µm), in order to facilitate comparisons with measurements in diameter from previous work [32].

5.2.7. Estrus synchronization of recipients, embryo transfer and pregnancy diagnosis

Detailed procedures for estrus synchronization of recipients and embryo transfer have been described [33]. Briefly, cyclic crossbreed heifers were synchronized in estrus by using an intravaginal progestagen device (PRID Alpha, Ceva Salud Animal, Barcelona, Spain) for 10 days, combined with a prostaglandin analog (Dynolytic, Pfizer, Madrid, Spain) injected 48 h before progestagen removal. Embryos cultured with or without 0.1% FCS up to Day-6, and thereafter individually cultured until Day-7, were selected for a preliminary

ET trial. Fresh or vitrified/warmed Day-7 produced blastocysts and expanded blastocyst were nonsurgically transferred to recipients in the cranial third of the uterine horn ipsilateral to corpus luteum under epidural anesthesia. The selection of recipients for fixed time ET required Day-7 progesterone (P4) serum levels >2.5 ng/mL and Day-0 P4 serum levels <2.0 ng/mL. Pregnancy was diagnosed by transrectal ultrasound scanning on Day-40 and Day-60.

5.2.8. Experimental design (Fig.5.1)

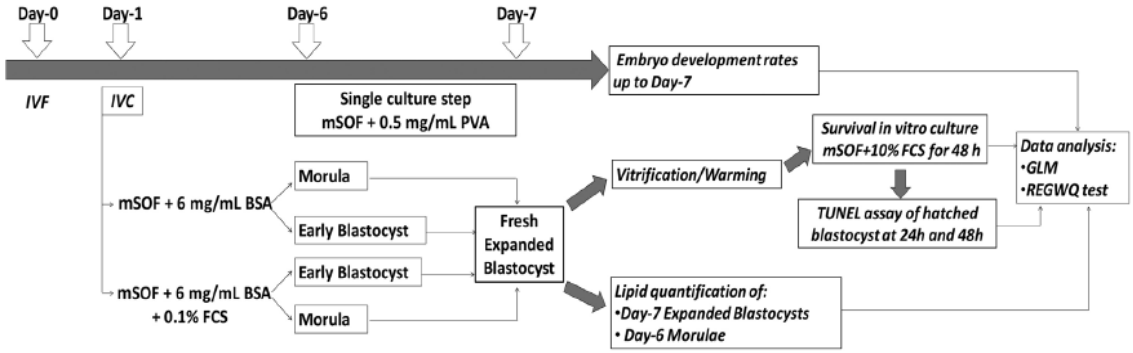

Fig. 5.1. Experimental design

5.2.8.1. Experiment 1: FCS (0.1%) supplementation and embryo development

The objective of experiment 1 was to determine the effects of 0.1% FCS in CM from Day-1 to Day-6 prior to a 24 h single culture without protein. After fertilization, zygotes were randomly cultured in two different conditions from Day-1 to Day-6: (i): mSOF + 6 mg mL^{-1} BSA, and (ii): mSOF + 6 mg mL^{-1} BSA with 0.1% FCS. On Day 6, morulae and early blastocysts from each of these two groups were selected and cultured individually without protein in 7 replicates. Blastocyst development was monitored on Day-7 (168 h PI)

5.2.8.2. Experiment 2: FCS (0.1%) supplementation and *in vitro* survival to cryopreservation

Experiment 2 aimed at assessing whether embryo survival to vitrification/warming after a 24 h single culture without protein was affected by previous culture from Day-1 to Day-6 with FCS. Excellent and good quality fresh expanded blastocysts were selected and vitrified in 7 replicates and warmed in 4 replicates. Re-expansion and hatching rates were monitored at 24 h and 48 h time points.

5.2.8.3. Experiment 3: FCS (0.1%) supplementation and apoptosis rates of vitrified/warmed Day-7 expanded blastocysts

The aim was to evaluate the percentage of apoptosis and total cell counts on hatched blastocysts produced in the experiment 2 after vitrification/warming (n = 48 embryos vitrified from 7 culture replicates and warmed in 4 replicates). Embryos were fixed at 24 h and 48 h and stained with -TUNEL and DAPI-assay in 4 replicates.

5.2.8.4. Experiment 4: FCS (0.1%) supplementation and lipid contents of Day-6 morulae and Day-7 expanded blastocysts

The lipid accumulation per embryonic cell was analyzed in fresh Day-7 expanded blastocysts (n = 62 embryos), as well as in fresh Day-6 morulae (n = 36 embryos) produced with or without 0.1% FCS. Morula is the earliest embryonic stage selected for single culture without protein. A total of 5 replicates were evaluated.

5.2.9. Statistical analysis

Data requiring normalization were arcsine transformed and analyzed using the Proc GLM module of SAS/STAT (version 9.2; SAS Institute Inc., Cary, NC). In experiment regarding embryo development up to Day-6, the fixed effects included were the presence of FCS in culture media from Day-1 to Day-6 and replicate. In experiments regarding development after protein deprivation, *in vitro* survival to vitrification/warming, apoptosis rates and lipid droplets evaluation, the fixed effects included were the presence of FCS in culture media from Day-1 to Day-6, embryonic stage on Day-6, interactions between FCS in culture media from Day-1 to Day-6 and embryonic stage on Day-6 and replicate. Least squares means and their errors (LSM ± S.E.M.) were estimated for each level of fixed effects with a significant F-value. The Ryan–Einot–Gabriel–Welsch Q-test was used to compare the raw means of the levels from the fixed effects.

5.3. Results

5.3.1. Embryo development

Development of embryos cultured in groups from Day-1 to Day- 6 can be observed in Table 5.1. On Day-6, early blastocyst rates from oocytes cultured with FCS (17.7 ± 1.4) were higher than the ones from oocytes cultured without FCS (6.3 ± 1.4) ($P = 0.022$). Individual development from Day-6 to Day-7 embryo is presented in Table 5.2. On Day-7, total blastocysts and expanded blastocyst rates were significantly higher in embryos from Day-6 early blastocyst than the ones from Day-6 morula, both with and without FCS prior to Day-6 ($P < 0.005$).

Table 5.1. Morula and early blastocyst rates on Day-6 after culture in mSOF + 6 mg mL^{-1} BSA in groups with or without 0.1% fetal calf serum (FCS).

		Day-6 (%)	
FCS	***n***	**Morula**	**Early blastocyst**
(-)	1133	40.9±1.5	6.3±1.4^{x}
(+)	908	38.3±1.5	17.7±1.4^{y}
P value		*0.709*	*0.022*

n = number of fertilized oocytes cultured. Data from 7 replicates (LSM ± s.e.m.). Superscripts $^{x, y}$ express significant differences.

Table 5.2. *In vitro* development of Day-6 morulae and early blastocysts produced in mSOF+ 6 mg mL^{-1} BSA in groups with or without 0.1% FCS, and after single culture without protein up to Day-7.

			Day-7 Blastocysts (%)	
FCS	**Day-6 stage**	***n***	**Total**	**Expanded**
(-)	Early Blastocyst	35	90.0±4.7^{x}	53.1±4.6^{x}
(-)	Morula	242	59.2±4.7^{y}	16.2±4.6^{y}
(+)	Early Blastocyst	96	94.0±4.7^{x}	50.3±4.6^{x}
(+)	Morula	187	37.6±4.7^{y}	7.6±4.6^{y}
P value			***<0.005***	***<0.005***

n = number of Day-6 morulae and early blastocysts cultured individually. Data from 7 culture replicates (LSM ± s.e.m.). Superscripts $^{x, y}$ express significant differences.

5.3.2. *In vitro* survival to vitrification and warming

After vitrification/warming, interaction between FCS and stage did not affect survival rates of Day-7 expanded blastocyst (P > 0.05), with 100% embryos surviving at 24 h in all treatments (Table 5.3). Interestingly, hatching rates of Day-7 embryos produced with FCS did not differ from embryos produced without FCS (P > 0.05). However, hatching rates at 24 h in EB-XB without FCS tended to be higher than in M-XB with FCS (P = 0.08). Such a tendency was not observed at 48 h, with all groups showing comparable hatching rates.

Table 5.3. *In vitro* survival after vitrification/warming of Day-7 bovine expanded blastocysts derived from morulae and early blastocysts produced in mSOF + 6 mg mL^{-1} BSA with or without 0.1% FCS from Day-1 to Day-6 after one-day single culture without protein.

				Re-expanded (%)		Hatched (%)	
Day-6 Stage	**FCS**	**Day**	***n***	**24h**	**48h**	**24h**	**48h**
Early blastocyst	(-)	7	30	100.0±4.9	93.2±8.9	58.3±10.5	53.0±14.6
Morula	(-)	7	57	100.0±4.1	84.6±7.4	30.6±8.7	50.2±12.2
Early blastocyst	(+)	7	67	100.0±4.5	92.3±8.2	31.8±9.6	51.3±13.5
Morula	(+)	7	26	100.0±4.6	90.8±8.3	28.1±9.7	47.8±13.6

n = number of Day-7 expanded blastocysts vitrified/warmed. Expanded blastocysts vitrified from 7 culture replicates and warmed in 4 replicates (LSM ± s.e.m.).

5.3.3. Apoptosis and total cell numbers

Total cell number and TUNEL-positive apoptosis percentage are shown in Fig. 5.2a with illustrative photomicrographs in Fig. 5.2b. Total cell numbers of hatched blastocysts after vitrification/warming were not affected by 0.1% FCS ($P > 0.05$), or by embryonic stage on Day-6 ($P > 0.05$). In contrast, FCS supplementation reduced cell counts in fresh expanded blastocysts examined in the lipid quantification study (+FCS: 109.3 ± 3.5 vs. - FCS: 119.4 ± 3.2; $P = 0.02$). FCS supplementation and embryonic stage on Day-6 did not affect either apoptosis rates of hatched blastocysts after vitrification/warming ($P > 0.05$).

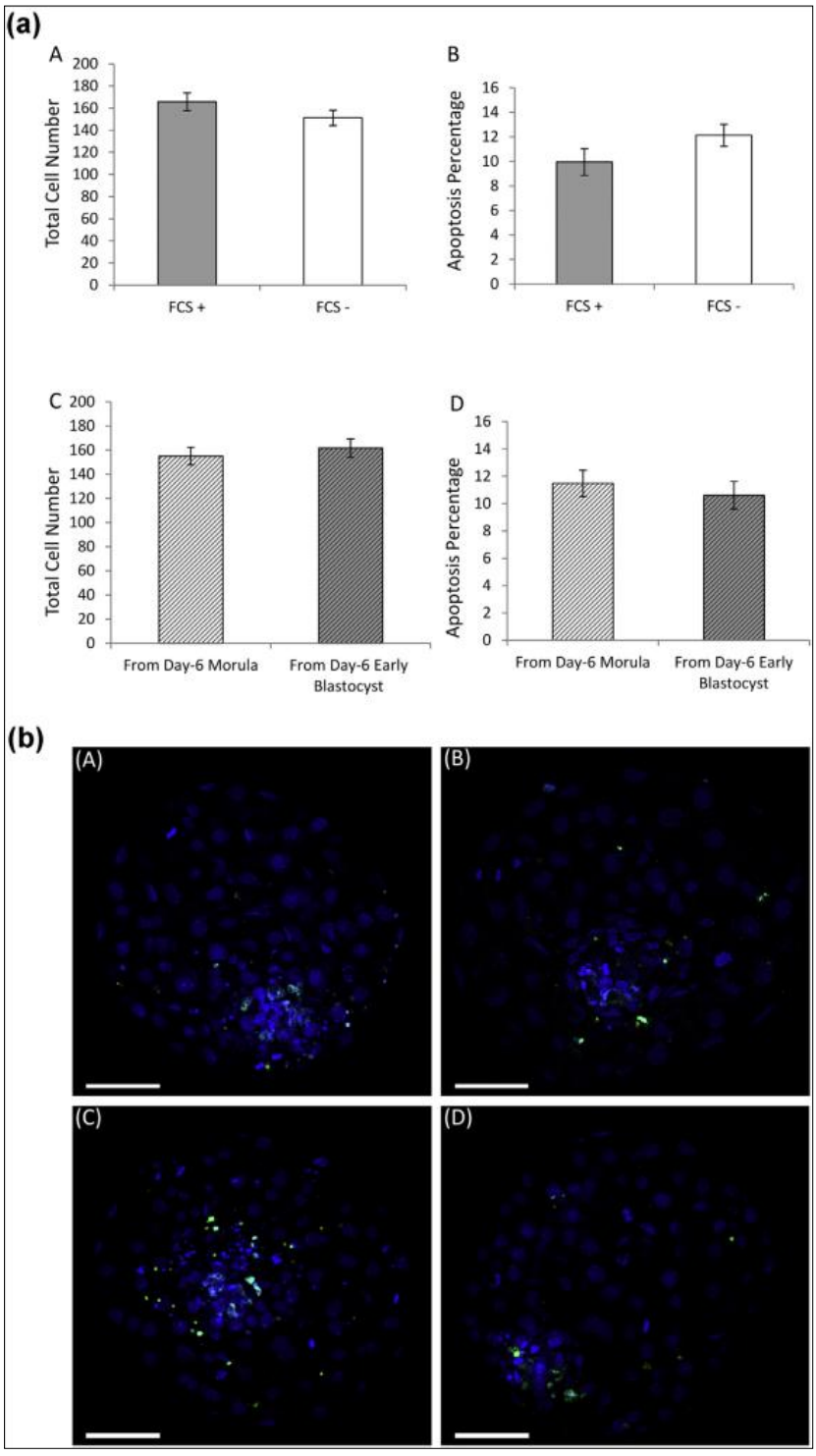

Fig. 5.2. (a) Effects of serum (FCS) in culture up to Day-6 and developmental stage (i.e., morula or early blastocyst) on Day-6, on total cell numbers (A, C) and apoptosis percentages (B, D) within hatched blastocysts surviving in culture after vitrification/warming of Day-7 expanded blastocysts. **(b)** Representative photomicrographs of single Z-axis confocal section of hatched blastocysts after vitrification/warming of expanded blastocysts produced in mSOF + 6 mg mL^{-1} BSA with FCS up to Day-6 (A) or without FCS up to Day-6 (B), and after one-day single culture without protein from Day-6 morula (C) or from Day-6 early blastocyst (D). Images showing TUNEL-positive cells in green and nuclear DAPI staining in blue. Scale bar 50 mm.

5.3.4. Lipid quantification study

Intracellular LD accumulation on Day-7 expanded blastocysts was higher with FCS than without FCS (84.5 ± 4.0 vs. 64.4 ± 3.6, respectively; P = 0.0002) (Fig. 5.3a). The embryonic stage on Day-6 affected cell counts and LD of Day-7 expanded blastocysts. Thus, M-XB showed higher cell counts than EB-XB (P = 0.01), and LD per cell in M-XB were more abundant than in EB-XB (P = 0.03). Fig. 5.3b shows 3D reconstructions from the confocal datasets that illustrate the LD distribution by size. When LD were arranged by size, FCS increased numbers of small, medium and large LD on Day-7 expanded blastocysts (P < 0.01). However, M-XB showed more medium and large LD per cell (P < 0.02 and P < 0.0004, respectively) than EB-XB. In the absence of FCS, EB-XB showed the highest cell counts (P < 0.05). As expected, Day-6 morula showed lower cell counts than the number of cells counts in the Day-7 expanded blastocyst different groups (P < 0.05). However, cell counts in Day-6 morula were similar, regardless of the embryos having been cultured in the presence or in the absence of FCS. Interestingly, Day-6 morulae cultured with FCS showed more total LD per cell than all other embryonic stages and conditions analyzed on days 6 and 7 (P < 0.001). In addition, Day-6 morulae cultured with FCS contained smaller LD than all the types of Day-7 expanded blastocysts analyzed (P < 0.001). However, the amounts of medium and large LD were similar between Day-6 morulae cultured with and without FCS and all types of Day-7 expanded blastocysts.

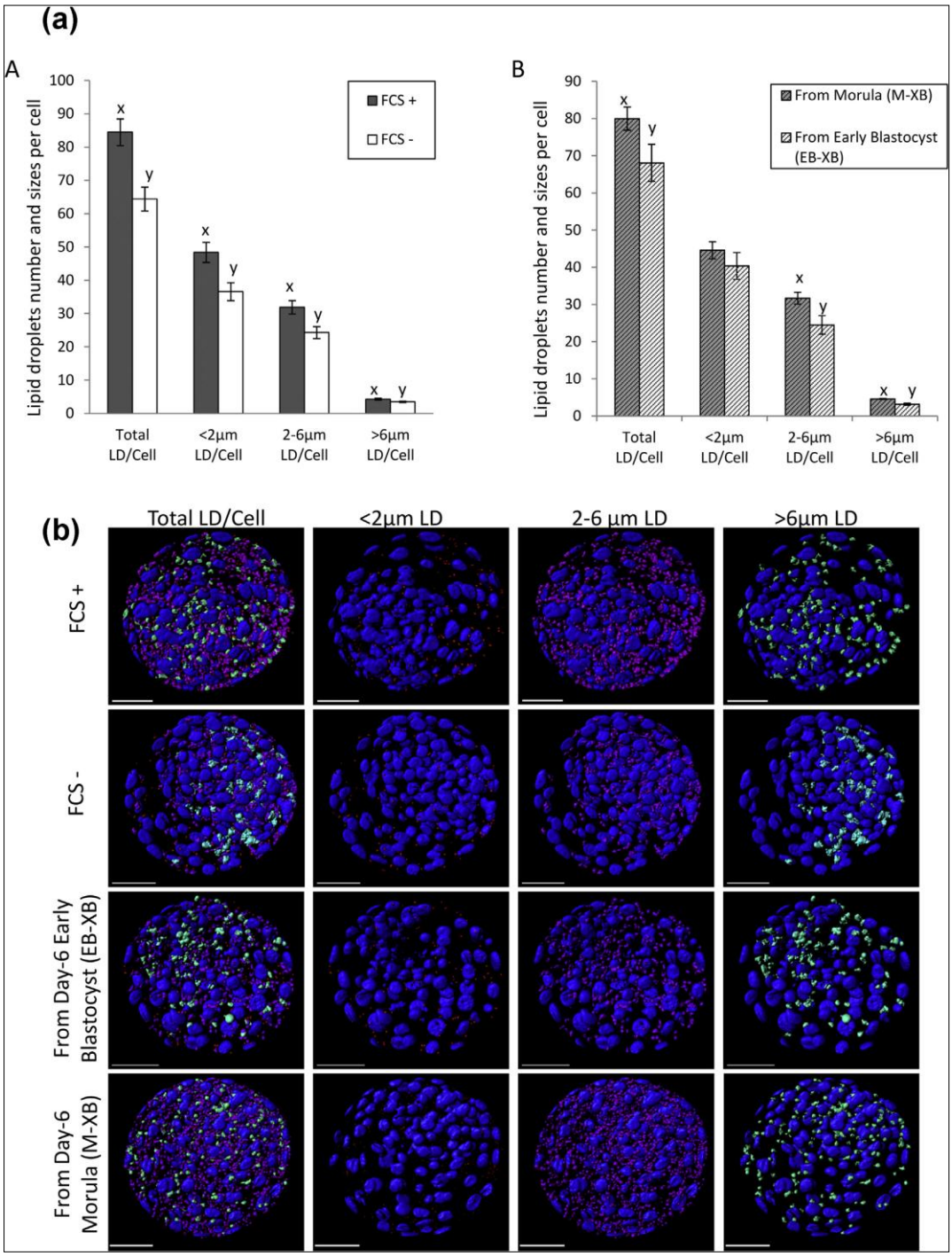

Fig. 5.3. (a) Lipid droplets number and sizes per cell counted in fresh expanded blastocysts from culture with FCS up to Day-6 (A; FCS +) or without FCS up to Day-6 (A; FCS-), from Day-6 morula (B; M-XB) or from Day-6 early blastocyst (B; EB-XB). LSM ± s.e.m. (x, y): $P < 0.03$. **(b)** Representative three-dimensional reconstructions (frontal view) of confocal datasets showing lipid droplets stained with Nile Red in fresh expanded blastocysts produced in mSOF + 6 mg mL^{-1} BSA with FCS up to Day-6 (FCS +) or without FCS up to Day-6 (FCS-), and after one-day single culture without protein from Day-6 morula (M-XB) or from Day-6 early blastocyst (EB-XB). Lipid droplets (LD) have been pseudocoloured according to their size: <2 mm (red), 2-6 mm (purple), >6 mm (green). Nuclei were stained with DAPI (blue). Scale Bars: 50 mm.

5.3.5. Embryo transfer and pregnancies

A first ET trial with Day-7 fresh and vitrified/warmed blastocysts (blastocyst and expanded blastocyst) transferred to recipients can be observed in Table 5.4. Day-7 blastocysts transferred were obtained from culture with or without FCS up to Day-6, followed by selection of morula, early blastocyst or blastocyst stage to one-day single culture without protein. Pregnancies were monitored on Day-40 and Day-60. Preliminary results showed that pregnancies were recorded at all stages analyzed, regardless of the stage on Day-6 (morula, early blastocyst or blastocyst) or the culture medium used up to Day-6 (with or without FCS).

Table 5.4. Descriptive Day-40 and Day-60 pregnancy status of heifers transferred with embryos cultured from Day-1 to Day-6 in mSOF + 6 mg mL^{-1} BSA, with or without 0.1% FCS and after one-day single culture without protein.

Culture media from Day-1 to Day-6	*n*	Day-6 Stage	Day-7 Stage	Status at ET	Day-40 Pregnancy	Day-60 Pregnancy
FCS + BSA	3	M	B	F	2/3	2/3
	2	M	XB	F	2/2	2/2
	2	M	XB	V	1/2	1/2
	1	EB	B	F	0/1	0/1
	3	EB	XB	F	2/3	2/3
	1	B	XB	F	1/1	1/1
Total	***12***				***8/12***	***8/12***
BSA	6	M	B	F	1/6	1/6
	2	M	XB	F	1/2	0/2
	2	M	XB	V	1/2	1/2
	1	EB	B	V	1/1	1/1
	2	EB	XB	F	1/2	1/2
	4	EB	XB	V	2/4	2/4
	2	B	XB	V	1/2	1/2
Total	***19***				***8/19***	***7/19***

ET: embryo transfer; N: number of Day-7 ET; M: Morula; EB: Early Blastocyst; B: Blastocyst. XB: Expanded Blastocyst; F: Fresh; V: Vitrified/warmed.

5.4. Discussion

In this study we describe how *in vitro* embryo culture in the presence of a very low amount of FCS improves early blastocyst rates on Day-6, which leads, after

single culture in absence of protein, to more expanded blastocysts on Day-7. The enrichment of CM with 0.1% FCS increased lipid accumulation in expanded blastocysts; however, their survival to vitrification/warming and their apoptosis rates were similar to BSA-produced embryos. Our previous results indicated that, only 10% of embryos reached the early blastocyst stage on Day-6, and the morula to expanded blastocyst development rates were lower without protein [9,10]. In this experiment, we improved early blastocyst rates by adding minute proportions of serum from Day-1 to Day-6, prior to protein removal. Thereafter, the early blastocyst stage on Day-6 led to increased yields of expanded blastocyst on Day-7, both with and without serum in culture. Our current study suggests that early blastocysts had lower substrate requirement than morulae in order to reach the expanded blastocyst stage. Post-compaction stages require more protein [34] and amino-acids [35] for their development than cleavage stages do. However, protein requirement seems to be strictly stage-dependent because early blastocysts develop and expand at high rates both with and without protein [9,10]. Kinetics studies in bovine embryos usually analyzed different stage endpoints, but not the same developmental endpoint reached by embryos that progressed from different earlier stages. Monitoring embryo development according to specific patterns appears to be more adequate than specific embryonic stages for selecting superior viability embryos [36]. Previous studies in our laboratory described the advantages of individual culture in simple medium [5–7] and how protein starvation improves embryonic viability [9,10]. Furthermore, one-day single culture without protein has allowed the identification of non-invasive and high accuracy embryonic sex biomarkers [8]. Supplementation of bovine embryo culture medium with serum, especially FCS,

is quite frequent. Thus, several studies show that the presence of serum may inhibit early cleavage even though later development up to the blastocyst stage was accelerated [20,23,34]. In this work, cleavage rates were not affected when CM was supplemented with 0.1% FCS. In addition, the use of FCS allowed selecting more expanded blastocysts for vitrification on Day-7. The lipid fraction present in serum could be incorporated into developing embryos [32] and affect the quality of resulting blastocysts [37]. Interestingly, the presence of serum prior to Day-6 did not affect *in vitro* survival rates of Day-7 expanded blastocysts after vitrification/warming. In this way, 100% embryos re-expanded at 24 h in all treatment groups, eliminating the pre- transfer culture, a time consuming and costly operation. This is consistent with our previous observations of 98% of vitrified/ warmed embryos re-expanding at 24 h after single culture without protein from Day-6 onwards [9]. We also observed that Day-7 expanded blastocysts produced with FCS tended to show reduced hatching rates at 24 h. Nevertheless, this can be interpreted as a delay, because similar hatching rates were observed in all treatment groups at 48 h. Further experiments will be necessary to determine if the delayed hatching induced by FCS can impact pregnancy rates after ET with vitrified/warmed embryos. In cows, an important goal is to improve blastocyst rates of Day- 7 *in vitro* produced embryos. It is generally accepted that Day-7 embryos, particularly at the expanded blastocysts stage, show higher pregnancy rates than Day-8 embryos and/or earlier embryonic stages [38,39,11]. Moreover, when embryo vitrification is a requirement, the embryo transfer with EB-XB results in calves with birthweight lower than M-XB [10], which avoids difficulties at birth and improves feto-maternal welfare. Apoptosis incidence in early embryos is an important parame-

ter for evaluation of embryonic health [40,41]. In addition, Sudano et al. [27] observed that the incidence of apoptosis in embryonic cells strongly correlates with embryo survival after cryopreservation. Apoptosis controls cell quality by removing damaged, impaired, nonfunctional, or even excessive cells [42]. However, a disproportionate incidence of cell death is associated with a reduction of embryonic viability [41,43–45]. In this study, neither FCS supplementation nor Day-6 embryonic stage (i.e. Morula or early blastocyst) increased apoptosis rates of hatched blastocyst after vitrification/warming. It has been shown that serum supplementation increases embryonic cells number [46,47], whereas others studies reported a reduction [43] of cells number. Although minimum numbers of embryonic cells are necessary for establishing pregnancy [48], the optimal cell amounts and distribution of inner cell mass and trophectoderm in the blastocyst are unclear. In this study, total cell counts in hatched blastocyst surviving vitrification/warming were unaffected by FCS supplementation or embryonic stage on Day-6. Interaction between FCS and embryonic stage did not affect lipid accumulation on Day-7 expanded blastocysts. However, FCS increased the numbers of intracellular LD and reduced cell counts on Day-7 expanded blastocysts. Cellular and molecular mechanisms of lipid accumulation in embryos produced *in vitro* are still poorly understood. However, reducing FCS concentration in culture was enough to decrease lipid contents and increase survival to cryopreservation [20]. In our previous experiments, we observed that the reduction in lipid contents typically observed in the transition from the morula to the expanded blastocyst stage in the presence of protein was more pronounced in the absence of protein. Therefore, both exogenous protein and Day-6 embryonic stage (i.e. Morula or

early blastocyst) affected lipid contents as observed at the Day-7 expanded blastocyst stage [10]. In this study, the absence of FCS in culture medium reduced intracellular LD, and lipid accumulation was greater in the Day-6 morula stage than in later stages, as observed by Sudano et al. [49]. The increased lipid contents in embryos cultured with FCS can be due to any of the following mechanisms: (I) Absorption of lipoproteins presents in serum [50]; (II) Activation of neo-synthesis of triglycerides in embryonic cells [51]; and (III) Modification of b-oxidation inside the mitochondria [32]. Therefore, once the oxidation-reduction state is changed, mitochondrial metabolism becomes modified and b-oxidation of lipid complexes is impaired, leading to high lipid accumulation [32,52,53]. Interestingly, Day-6 embryonic stage affected lipid contents in the Day-7 expanded blastocyst stage. Day-7 expanded blastocysts exhibited increased LD accumulation when embryos were produced from Day-6 morula stage in contrast to early blastocyst. With the onset of morula compaction and during blastocyst formation, energy demands, measured as oxygen and nutrient consumption, increase dramatically. We proposed that, contrary to the blastocyst, the ability to use oxygen in the morula is still limited, which may hinder the lipid catabolism [10]. The heterogeneity of LD in size, location, and associated proteins within a given cell or tissue, and between different tissues, suggests that subpopulations of LD have most likely specialized functions in lipid storage and metabolism [54]. The differences in the LD sizes in bovine embryos produced *in vitro* have been previously described [32,55,56,10]. Lipogenesis associates with the smallest granules, which converge to form larger ones by different mechanisms [57]. Thus, FCS led to Day-6 morula containing larger amounts of small and total LD. The embryos

produced with FCS accumulated high LD amounts up to Day-6, which could not be reduced after one-day protein removal to the same levels as embryos produced in BSA. Moreover, FCS supplementation increased total LD on Day-7 expanded blastocyst, regardless of LD sizes. Finally, *in vitro* embryos cultured with FCS (0.1%) up to Day-6 have been transferred, and effective pregnancies were obtained. These preliminary results suggest that the presence of low FCS concentration prior to individual culture system on Day-6, is compatible with pregnancy under a variety of embryonic transitions in conditions that did not differ from BSA. Long-term effects of minimal concentration of serum will be evaluated in future studies. In conclusion, very low concentration of FCS allowed obtaining higher early blastocyst rates on Day-6, which, after one-day single culture without protein, led to improved expanded blastocyst rates. In terms of survival to cryopreservation, apoptosis rates and lipid contents, the quality of expanded blastocysts produced with FCS compares well with BSA. Even though long-term effects associated with birth weight and calf phenotype must be confirmed, our preliminary studies show that FCS do not reduces the viability of embryos to produce pregnancies.

5.5. References

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6 | CAPÍTULO IV

Hepatoma-derived growth factor: Protein quantification in uterine fluid, gene expression in endometrial-cell culture and effects on *in vitro* embryo development, pregnancy and birth

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Theriogenology 2017. *96, 118-125*

Project support was provided by the Spanish Ministry of Economy and Competitiveness -MINECO-, projects AGL2016-78597-R and AGL2015-66145-R. Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013-2017 (GRUPIN 14-114) and FEDER. AM was supported by Secretaría Nacional de Educación Superior, Ciencia, Tecnología e Innovación -Ecuador - II Fase 2013. The authors are members of the COST Action 16119, *In vitro* 3-D total cell guidance and fitness (Cellfit).

Abstract

Hepatoma-derived growth factor (HDGF) is present in the endometrium of cows and other mammals. Recombinant HDGF (rHDGF) improves bovine blastocyst development *in vitro*. However, specific culture conditions and essential aspects of HDGF uterine physiology are yet unknown. In this work we quantified total HDGF protein in uterine fluid (UF) by multiple reaction monitoring (MRM), and analyzed effects of rHDGF on specific embryonic stages with Day-6 bovine embryos cultured *in vitro* with and without BSA, and on pregnancy viability and calf phenotypes after embryo transfer to recipients. In addition, mRNA abundance of HDGF in endometrial cells co-cultured with one male or one female embryo was quantified. In the presence of BSA, rHDGF had no effect on blastocyst development; however, in BSA-free culture rHDGF mainly promoted development of early blastocysts in contrast with morulae. As the presence of HDGF contained in commercial BSA replacements was suspected, western blot confirmed HDGF identification in BSA both with and without fatty acids. Total HDGF quantified by MRM tended to increase in UF without vs. UF with embryos ($P = 0.083$). Pregnancy and birth rates, birth weight and calf measurements did not differ between embryos cultured with rHDGF and controls without rHDGF. However, HDGF abundance in cultured epithelial, endometrial cells tended to increase ($P < 0.08$) in culture with one male embryo. rHDGF acts selectively on specific embryonic stages, but care should be taken with specific macromolecular supplements in culture. The endometrial expression of HDGF can be regulated by the embryonic sex. The use of rHDGF is compatible with pregnancy and birth of normal calves.

6.1. Introduction

Growth factors (GFs) produced by the reproductive tract can improve *in vitro* bovine embryo development, and/or often pregnancy rates and survival to term. Reports include Dickkopf, epidermal growth factor, insulin-like growth factor 1, granulocytemacrophage colony-stimulating factor, hyaluronan, fibroblast growth factor 2, activin, and platelet-activating factor [1-9]. Hepatoma-derived growth factor (HDGF) is a 240 amino-acid protein isolated from supernatants of human hepatoma cells in culture [10]. Surface expressed nucleolin has recently been identified as a HDGF receptor [11], and HDGF stimulates cell proliferation in fibroblasts, endothelial cells and hepatoma cells [12]. In the cow, HDGF is resent in the endometrium and uterine fluid (UF) during early pregnancy [13,14], as well as in the endometrium and conceptus in horses [15] and in the UF of the tammar wallaby [16]. Interestingly, in cattle, changes induced in the uterine HDGF by embryos [13] are not reflected as mRNA transcription and protein abundance in endometrial cells [14] by which up-regulation of HDGF in UF could be dependent on post-transcriptional modifications, as previously postulated to occur in the uterus [17]. Notably, unlike the absence of detectable transcription in response to embryos in vivo, cultured endometrial cells transcribe *in vitro* in response to one embryo [18]. Recombinant HDGF (r-HDGF) improves blastocyst development and cell proliferation [14]. HDGF may act by both autocrine and paracrine mechanisms to promote early embryo development, and effects of rHDGF are strictly time and/or stage dependent (i.e. it stimulates embryo growth within Day-6 but not Day-5 morulae) [14]. Within *in vitro* embryo culture, similar stage- or time-dependent effects also occur with other GFs, resulting in altered blastocyst development and quality

[6,7,19,20] and, ultimately, improved pregnancy and birth rates [1,20]. In cultures with synthetic oviduct fluid (SOF), vitrified/warmed, Day-7 expanded blastocysts resulting of Day-6 morulae or early blastocysts deprived of protein yield lower miscarriage and improved birth rates upon transfer to recipients [21]. Interestingly, Day-6 morulae and early blastocysts mobilize lipid stocks differently in response to protein deprivation, and the Day-6 early blastocysts stage yields lighter calves than Day-6 morula when their cognate Day-7 vitrified expanded blastocysts are transferred [22]. Therefore, r-HDGF in culture could exert different stimulation on morulae and/or early blastocysts. Protein supplements used in bovine embryo culture are normally impure and can induce confounding effects when testing single molecules. Serum and its extracts, as bovine serum albumin (BSA), may carry GFs, cytokines, amino-acids, steroids and a variety of contaminants [23]. Cold-precipitated BSA exists as an embryo tested product, and more refined forms of BSA (e.g., essentially fatty-acid free -FAF-) are also used in embryo culture. Effects of exogenously added GFs can be masked not only by serum or its extracts, but also by autocrine and paracrine interactions (group embryo culture). The effects of HDGF on *in vitro* embryo culture were demonstrated both in group and single culture in chemically defined conditions [14], but it is unknown yet whether culture in medium with BSA would offer similar performance. Endometrial cells are responsive to embryonic sex early in development in vivo [24]. Therefore, endometrial HDGF could also show a sexually dimorphic transcription.

The objectives of this work were: 1) Total quantification of HDGF protein in UF by MRM, a technique that can be programmed to recognize peptides typically present in any protein isoform [25]; 2) Identifying the developmental effects of

rHDGF on specific embryonic stages, both in semi-defined (BSA) conditions and in chemically defined conditions; 3) Analyzing the presence of HDGF in commercial BSA and BSA-FAF extracts used in embryo culture; 4) Analyzing endometrial transcription for HDGF in response to one male or female embryo in cultured endometrial cells; and 5) Determining whether embryos produced with rHDGF can establish pregnancy and reach birth with production of normal calves.

6.2. Material and Methods

All experimental procedures were approved by the Animal Research Ethics Committee of SERIDA (Agreement 02/02/2012), in accordance with the European Community Directive 86/609/EC. Ovaries were collected from cows slaughtered in commercial abattoirs (Matadero de Leon and Matadero de Guarnizo -Cantabria-, mostly for Holstein ovaries, and Matadero de Tineo, for Asturiana de los Valles ovaries (Spain). Ovaries were transported to the laboratory in NaCl solution (9 mg mL^{-1}) with streptomycin sulfate, 100 mg ml^{-1}, penicillin, 100 IU mL^{-1} and maintained at 25-30 ºC. All reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated.

6.2.1. Embryo production

In vitro-produced (IVP) embryos were obtained as described [26] with minor modifications. Antral follicles (3-8 mm in diameter) were aspirated through an 18-g needle connected to a syringe. Aspirated fluid was expelled into dishes containing holding medium (HM) TCM199 (Invitrogen, Barcelona, Spain), 25 mM HEPES and BSA 0.4 g L^{-1}, and oocytes were searched under

stereomicroscope. Oocytes with more than three layers of compact cumulus cells with homogenous cytoplasm were selected. For *in vitro* maturation (IVM), cumulus oocyte complexes (COCs) were washed twice in maturation medium (MM) consisting of TCM199 NaHCO3 (2.2 g L^{-1}) supplemented with 10% (v/v) fetal calf serum, 1:5 mg mL^{-1} of porcine FSH-LH (Stimufol; ULg FMV, Liege, Belgium) and 1 mg mL^{-1} 17 b-estradiol. COCs were cultured (n = 30-50) into a four-well dish with 500 mL of IVM medium for 22-24 h at 38.7 ºC under 5% CO^2 with saturated humidity.

After IVM, oocytes were subjected to *in vitro* fertilization (IVF; Day 0) with frozen/thawed sex-sorted or non sex-sorted spermatozoa from Holstein or Asturiana de los Valles breeds following described procedures [26,27], respectively. For experiments entirely *in vitro* or for transient embryo transfer to the uterus (Day-6 to Day-8), IVF was performed with non-sorted spermatozoa from a single bull. However, for transfer to recipients for pregnancy, embryos were produced from oocytes both from slaughterhouse ovaries or collected by Ovum-Pick-Up (OPU) following described procedures [28], and fertilized with non sorted (N = 3 bulls) and female sex-sorted spermatozoa (N = 3 bulls). COCs were washed three times in HM in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 mg mL^{-1}; Calbiochem, La Jolla, CA, USA). For IVF, oocytes and sperm cells were incubated together for 18-20 h at 38.7 ºC in an atmosphere of 5% CO_2 with saturated humidity. Subsequently, cumulus cells were detached using a vortex, and presumptive zygotes were cultured in modified synthetic oviduct fluid (mSOF) containing amino acids (BME Amino Acids Solution), 45 mL mL^{-1} and MEM Non-essential amino-acid Solution (SIGMA, Madrid, Spain), 5 mL mL^{-1}),

citrate, myo-inositol, and BSA (6 g L^{-1}), as previously described [21]. Until Day-6, embryos were cultured in groups (N = 35-45) in droplets of mSOF (1-2 mL per embryo) layered down mineral oil. *In vitro* culture was carried out at 38.7 ºC, 5% CO_2, 5% O_2 and saturated humidity. After Day-6 embryos were cultured singly using different procedures as described below.

6.2.2. Vitrification of embryos for transfer

Vitrification procedures have been described in detail [26]. Briefly, Day-7 excellent expanded blastocysts were vitrified in two steps with fibre-plugs (CryoLogic Vitrification Method; CVM). Vitrification solutions contained DMSO, ethylene-glycol, and sucrose. Samples were vitrified by touching the surface of a chilled block placed in LN_2 with the fibre plug hook that carried the drop with the embryo. Embryos were warmed by direct immersion of the fibre plug end in sucrose solution in one-step [29]. Subsequently, embryos were washed twice in Embryo Preservation Medium (IMV Technologies, France) and loaded in straws for transfer to recipients. *In vitro* development of vitrified/warmed embryos cultured with and without protein was already reported [21].

6.2.3. Animals and embryo transfer (ET) for uterine fluid collection

Detailed procedures were described [13]. Briefly, cyclic Holstein heifers (N= 14) were synchronized in estrus with an intravaginal progestagen device combined with a prostaglandin analogue. Day 0 was considered a fixed time 48 h after progestagen removal, in coincidence with the IVF onset in the laboratory. Day-6 IVP embryos (n = 50), or vehicle (Sham transfer) were transferred into the uteri

of each estrus synchronized Holstein heifer (n = 14) at non consecutive cycles under epidural anesthesia.

On day 8, prior to being flushed, recipients were monitored to verify the presence of a corpus luteum in the expected ovary. Uterine flushings were performed using silicon Foley catheters loaded to flush the two cranial thirds of the horn ipsilateral to the corpus luteum. All recipients were first flushed with 45 mL PBS. Recovery of diluted uterine fluid (UF) was performed by aspirating with a 50 mL syringe (Becton-Dickinson, Zaragoza, Spain), only while a steady flow could be achieved. If embryos were transferred, the flushed fluids were directly filtered through an Em-Com device in order to retain the embryos, while the outlet fluid was collected in a tube and directly added with 10 mL mL^{-1} protease inhibitor (Protease Arrest; GE Healthcare, Madrid, Spain). Those recipients transferred with embryos were next extensively flushed with PBS +1 mg mL^{-1} polyvinyl-pyrrolidone (PVP; mw 40000; P0930). Embryos were identified using a stereomicroscope and rapidly separated from fluids to be used in other experiments. Collected UF with embryos contained on average n = 43.1 ± 5.2 total and n = 34.1 ± 3.7 % viable embryos (as proportions of transferred morulae). UF was centrifuged (2000g) at 4 ºC, aliquoted and stored at -145 ºC. Concentrations of P4 lower than 2.0 ng mL^{-1} on Day- 0 and higher than 3.5 ng mL^{-1} on Day-6 and Day-8 were recorded in all animals whose UFs were collected for analysis.

6.2.4. Animals and embryo transfer for pregnancy and birth

Detailed procedures were described [30]. Briefly, Holstein and Asturiana de los Valles heifers were synchronized in estrus by using an intra-vaginal

progestagen device (PRID Alpha) for 10 days combined with a PG analogue (Dynolitic, Pfizer, Madrid, Spain) injected 48 h before progestagen removal. Prior to transfer, Day-7 expanded blastocysts that had been vitrified at 170-172 h PI were warmed and allowed to re-expand for 1-2 h *in vitro*. On cycle Day 7 (225 ± 1.5 h after progestagen removal; fixed time) blastocysts were non-surgically transferred to recipients into uterine horn ipsilateral to CL under epidural anesthesia. Pregnancy was diagnosed on Day 40 and on Day 62; birth rates were monitored in recipients that were made pregnant after 1 to 4 ETs. At birth, body weight (BW), height at withers (HW), thorax circumference (TC), gestation length (GL) and daily weight (BW/GL), were recorded in the calf; BW was also measured in the mother. Plasma P4 was measured on Day 0 and Day 7 (5e6 h before ET) in blood samples taken up into EDTA vacuum tubes via coccygeal vein puncture. An ELISA test operating on a 0e40 ng/mL scale (DRG Diagnostics, Marburg, Germany) was used. The test was sensitive starting from 0.5 ng mL^{-1} and cross-reactivity from steroids other than P4 was less than 1%. Intra- and inter-assay coefficients of variation were 6% and 7%, respectively.

6.2.5. Multiple reaction monitoring (MRM) for HDGF contents in uterine fluid

HDGF abundance in UF was analyzed in UF collected on Day-8 by programming peptides to evaluate total protein content by multiple reaction monitoring (MRM). Samples of UF were concentrated, and protein was precipitated and suspended in ammonium bicarbonate. Protein (20 mg) was reduced with DTT, trypsin-digested and desalted. Five proteotypic peptides for HDGF were selected with MRM Pilot software (ABsciex), with 5 transitions

programmed for each peptide. A control, unrelated synthetic peptide was spiked as an internal standard. The sequences of the 5 selected peptides did not include phosphorylable residues (Table 6.1), as reported in Uniprot for human HDGF (i.e. excluding coverage of serine (S): 135, 133, 165, 199; 202, 206, 238 and threonine (T): 184, 200 residues).

Table 6.1. Sequence of peptides from bovine hepatoma-derived growth factor detected in the multiple reaction monitoring study and their position in the protein.

Bovine peptide sequence	Amino acid position
C*GDLVFAK	12-19
ASGYQSSQK	97-105
LVIDEPTK	139-146
EAEDLEGEEK	171-180
GPPQEEEEEEEEEEAAKE	209-226

The area of the larger transition for the control peptide was used to normalize the area values of each other peptide. Peptides used to calculate relative protein concentration were evaluated for signal to noise ratio, reproducibility and validated with synthetic peptides. MRM experiments were performed on a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (ABsciex) equipped with an Eksigent 1D þ plus nanoLC chromatographic system. Data analysis was performed with Analyst 1.5.2 and MultiQuant 2.0.2 software (ABsciex). The area of most abundant transition for each analyzed peptide was used for relative quantitation.

6.2.6. Identification of HDGF in BSA supplements by western blot

Two types of BSA used in embryo culture were tested for the presence of HDGF. BSA A-3311, (batch no. 089K14281V, embryo culture tested), and BSA fatty-acid free (A-6003; batch SLBD6158V; >96% fatty-acid free). Immunoblotting was performed as described previously [13]. Briefly, 2.5 mg/sample was electrophoresed on 12% SDS-PAGE gels. Proteins were then

transferred electrically onto a PVDF membrane (Millipore, Billerica, MA, USA), which was incubated with a mouse polyclonal antibody against human HDGF (predicted to work with bovine according to supplier; Abcam 43668; diluted 1:1500). After washing, the membrane was incubated with the required secondary antibody (goat antimouse/ HRP conjugate, NA931-1ML; Amersham Pharmacia Biotech) at 1:4000 dilution and detected using the Pierce ECL detection kit (Thermo Scientific, Waltham, MA, USA). Protein levels were not quantified.

6.2.7. Endometrial cell and embryo co-culture

Endometrial cells were obtained from early estrus cycle slaughterhouse uteri from heifers as described [18]. Briefly, ipsilateral uterine horns were excised, washed with 70% ethanol and cultured with 0.75% EDTA in PBS at 37 ºC during 60min. Epithelial cells (EC) and stromal cells (SC) were collected separately.

For EC collection, the horn was longitudinally excised and the inner surface scrapped. Cells were collected in DMEM-F12 (D8437) with 10% FCS and antibiotics, and incubated for 3 h. Subsequently, the cell mixture was centrifuged 8 min at 150g, and the pellet was washed before seeding in cell-culture inserts 1.0 mM pore size, 6.5mm in diameter (Millipore, Madrid, Spain) coated with diluted Matrigel (1:2) in DMEM-F12. Cells were observed daily and the first renewal was performed at 48 h or 72 h, depending on cell growth rate monitored. Subsequent renewals were performed every 48 h after the first renewal.

For SC collection, endometrial tissue strips were minced and digested in 0.5% collagenase (C5138) in DMEM/F12 for 3 h. The suspension was filtered through a 250 mM mesh and subsequently through a nylon 30 mM Millipore filter. The filtered samples were centrifuged 8 min at 150g, and re-suspended in 5 mL DMEM/F12 with 10% FBS and antibiotics. Cells were seeded in the basal compartment of 24-well plates. Culture medium was renewed first after 18 h culture and subsequently every 48 h.

For embryo-endometrial co-culture, EC cultures in inserts were placed in 24-well plates onto SC cultures. Only replicates with 100% confluence within SC and >90% confluence in EC 7 days after seeding where selected for embryo co-culture. Prior to embryo loading in EC, the cells were washed and cultured with serum-free TCM-199. One Day-6 IVP morula was loaded in the apical side of the EC, and co-cultures were maintained in serum free TCM-199 medium for 48 h. At the end of the co-culture period, the embryos were assessed for development stage and morphology and sexed by amelogenin gene amplification [31]. After removing culture medium, EC and SC were collected for gene expression.

6.2.8. Gene expression of HDGF in epithelial and stromal endometrial cell cultures

Procedures have been described [18]. Briefly, EC in inserts and SC in basement 12-well plates were collected after 8 days of culture. Cells were extracted with Trizol (Invitrogen, Madrid, Spain e #15596-026) added in volumes of 250 mL (EC in inserts) or 500 mL Trizol (SC in the basal compartment) for 2 min. Upon collection, Media with lysed cells were

centrifuged at 4 ºC 10 min and 12,000g. The supernatant (approximately 200 mL, EC; over 500 mL, SC) was loaded in Eppendorf tubes, snap frozen in LN_2 and stored at -80 ºC.

Total RNA was extracted from EC and SC using TRIsure (Bioline, Reus, Spain) and Direct-zol RNA miniprep kit (Zymo Research, E1009). Subsequently, RNA was eluted with Tris-HCl. Immediately after extraction the RT reaction was carried out [32] using poly (T) primers, random primers, and MMLV High Performance Reverse Transcriptase enzyme (Epicentre Technologies Corp., Madison, U.S.A.) to prime the RT reaction and to produce cDNA. Tubes were heated to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. Tubes were then incubated at 25 ºC for 10 min to promote annealing of random primers, followed by 37 ºC 60 min to allow the RT of RNA. Finally, the samples were incubated at 85 ºC for 5 min to denature the enzyme.

qPCR reactions were carried out in duplicate in the Rotorgene 6000 Real Time Cycler TM (Corbett Research, Sydney, Australia) by adding 5 ng of each sample to the PCR mix (GoTaq qPCR Master Mix, Promega Corporation, Madison, USA) containing the specific primers selected to amplify the selected gene. qPCR reactions were run at 95 ºC for 3 min, 40 cycles of 94 ºC for 15 s, 56 ºC for 30 s and 72 ºC for 15s, and 10 s for fluorescence acquisition. Reactions were carried out in duplicate in the Rotorgene 6000 Real Time Cycler TM (Corbett Research, Sydney, Australia) by adding 5 ng of each sample to the PCR mix (GoTaq qPCR Master Mix, Promega Corporation, Madison, USA) containing specific primers for HDGF (Hepatoma derived growth factor; Accesion NO: NM_175832.2; forward primer: GACCCACGAGACGGCATT;

reverse primer: GCTGAACCCTTTCCTCTTGTTG; product length: 96 bp). Fluorescence was acquired in each cycle at temperature higher than melting temperature of primer dimers (84 ºC). Then, the threshold cycle was determined for each sample. The comparative cycle threshold (CT) method was used to quantify expression levels and values were normalized to the endogenous, housekeeping control (H2AFZ; Histone; Accesion NO: NM_174809; forward primer: AGGACGACTAGCCATGGACGTGTG; reverse primer: CCACCACCAGCAATTGTAGCCTTG; product length: 208 bp). The ΔCT value was determined by subtracting the mean housekeeping CT value for each sample from HDGF CT value of the sample. Fold changes in the relative gene expression of HDGF were determined using the formula 2-ΔΔCT [33]. Primers were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primersblast/) to span exon-exon boundaries.

6.2.9. Experimental design

6.2.9.1. Experiment 1: development of individually cultured morulae and early blastocysts in the presence of rHDGF

On Day 6 of IVC, good-quality morulae and early blastocysts were selected and cultured individually for 24 h in 12µL drops of mSOF + with (6 mg mL^{-1} BSA) or without protein (0.5 mg mL^{-1} polyvinyl-alcohol, PVA, was added to replace BSA) under mineral oil. Cultures with PVA were a retrospective analysis of published development [14]; cultures with BSA were made on purpose. Culture media with BSA or PVA contained whether or not r-HDGF in a concentration of 100 ng mL^{-1} [14]. Blastocyst development was monitored for 24 h (Day 7) and 48 h (Day 8).

6.2.9.2. Experiment 2: targeted HDGF protein quantification in UF and BSA supplements

After flushing, recovered UF was aliquoted, stored at -145 ºC and processed to quantify HDGF by MRM and WB. This experiment was repeated with N = 10 animals, each providing 1 Sham Transfer and 1 embryo transfer sample. Subsequently, as HDGF had no effect on embryo development in BSA containing medium, the presence of HDGF was analyzed by WB in two commercial BSA preparations used in embryo culture.

6.2.9.3. Experiment 3: influence of embryonic sex on gene expression of HDGF in cultured endometrial cells

HDGF relative expression was quantified in EC and SC cultured for 48 h with one single male embryo, one single female embryo or no embryos (control).

6.2.9.4. Experiment 4: pregnancy rates and birth measurements after transfer of embryos produced with rHDGF

Day-7 expanded blastocysts derived from Day-6 morulae and early blastocysts cultured individually with or without 100 ng mL^{-1} r-HDGF in medium without protein (i.e. PVA), were transferred to recipients as fresh or vitrified warmed. Pregnancy was monitored by ultrasound on days 40 and 62, and miscarriage rates were controlled until birth. At birth, body weight, height at withers, thorax circumference, gestation length and average daily gain weight were measured in calves.

6.2.10. Statistical analysis

Data requiring normalization were log transformed and analyzed using the Proc GLM module of SAS/STAT (version 9.2; SAS Institute Inc., Cary, NC). For embryo development rates, treatment (with or w/o HDGF), embryonic stage on Day-6 (morula and early blastocysts) were fixed effects; replicate was considered a random effect. For protein quantification in UF, fixed effects were cow, presence of embryos (i.e., ET vs. sham ET), analyzed gel (in the WB study), and replicate (i.e. round at which UF samples were collected). HDGF identification in BSA samples was qualitative. For pregnancy and birth rates, the effects considered were treatment (with or w/o HDGF), embryonic stage on Day-6 (morulae and early blastocysts) and ET round (replicate). For body measurements and birth weight, the fixed effects included were embryonic stage on Day-6, presence of HDGF in culture, bull, calf sex and recipient; season was a random effect. Least squares means and their errors (±SEM) were estimated for each level of fixed effects with a significant F-value. The Ryane-Einote-Gabriele-Welsch Q-test was used as a post-hoc test to compare the raw means of the levels from the fixed effects ($P < 0.05$).

In the gene expression study, the effects considered were embryonic sex (male, female or no embryo), biological replicate (i.e. uterus sample) and technical replicate (repeated PCR analysis). Data were analyzed with software package SigmaStat 3.5 (Jandel Scientific, San Rafael, CA, USA). Fluorescence intensity of epithelial and stromal cells and relative mRNA abundances for candidate genes were compared.

6.3. Results

6.3.1. Embryo development

In the presence of protein (i.e. BSA; Table 6.2), Day-6 morulae and early blastocysts were not affected by HDGF in their blastocyst development rates on days 7 and 8. Early blastocysts showed higher blastocyst development rates than morulae. In the absence of protein (Table 3), development rates of Day-7 blastocysts grown from Day-6 early blastocysts with HDGF (98.7 ± 9.4) were twice those than without HDGF (49.7 ± 8.7) ($P < 0.01$). Blastocyst rates with HDGF were numerically higher in all stages analyzed on Day-7 and Day-8 for Day-6 morulae and early blastocysts over their cognate non-treated embryos, although such differences were not significant.

6.3.2. HDGF contents in uterine fluid

For MRM, We analyzed N = 10 UF samples that contained embryos and N = 10 UF samples obtained after sham transfer. Proteotypic peptides programmed for HDGF were N = 5. Out of them, the peptide LVIDEPTK was identified in all samples analyzed and showed the best signal to noise ratio, reproducibility and validation with a synthetic peptide. Relative abundance of HDGF between UFs containing or not containing embryos tended to differ (2.85 ± 0.6 vs. 4.43 ± 0.6; respectively; $p = 0.083$).

Table 6.2. *In vitro* development of bovine morulae and early blastocysts cultured individually from day 6 to day 8 in droplets of synthetic oviduct fluid with BSA containing 100 ng mL^{-1} recombinant HDGF (rHDGF).

Treatment			Day-7 Blastocysts (%)			Day-8 Blastocysts (%)			
Stage	**[rHDGF]**	***n***	**Total**	**Blastocysts**	**Expanded**	**Total**	**Blastocysts**	**Expanded**	**Hatched**
Morula	0	184	79.5±3.3^{x}	57.3±3.3^{x}	23.4 ±8.7^{x}	81.6±3.0^{x}	66.6±2.8^{x}	49.9±7.1^{a}	4.3±7.9
Morula	100	187	68.6±3.3^{x}	54.4±3.3^{x}	21.3±8.7^{x}	76.0±3.0^{x}	66.1±2.8^{x}	45.6±7.1^{ya}	5.7±7.9
Early Blastocyst	0	20	100.4±3.6^{y}	100.7±3.5^{y}	74.7±9.4^{y}	101.2±3.3^{y}	101.5±3.0^{y}	88.0±7.7^{b}	22.8±8.6
Early Blastocyst	100	17	100.8±4.2^{y}	101.7±4.1^{y}	44.3±10.9	100.9±3.8^{y}	101.4±3.5^{y}	76.1±8.9^{b}	12.6±10.0

Data were collected from 10 replicates and expressed as proportions (LSM±SEM) of Day-6 cultured embryos (*n*).
Values in the same column with different letters show significant differences: ^{x,y} ($p < 0.01$); ^{a,b} ($p < 0.05$).

Table 6.3. *In vitro* development of bovine morulae and early blastocysts cultured individually from day 6 to day 8 in droplets of synthetic oviduct fluid with PVA containing 100 ng mL^{-1} recombinant HDGF (rHDGF).

Treatment			Day-7 Blastocysts (%)			Day-8 Blastocysts (%)			
Stage	**[rHDGF]**	***n***	**Total**	**Blastocysts**	**Expanded**	**Total**	**Blastocysts**	**Expanded**	**Hatched**
Morula	0	184	51.6±5.6^{x}	30.1±8.1^{x}	12.7 ±8.6^{a}	62.2±7.4	49.2±8.1^{a}	34.4±7.6^{x}	3.7±6.0
Morula	100	183	63.1±5.6^{x}	45.4±8.1^{x}	17.1±8.6^{a}	75.5±7.4	66.0±8.1	48.5±7.6	3.1±6.0
Early Blastocyst	0	20	90.3±6.1^{y}	49.7±8.7^{x}	46.7±9.3	90.4±8.0	83.3±8.7^{b}	57.6±8.3	11.0±6.5
Early Blastocyst	100	20	100.0±6.5^{y}	98.7±9.4^{y}	56.6±10.0^{b}	89.3±8.7	89.3±9.4^{b}	81.3±8.9^{y}	12.5±7.0

Data were collected from 10 replicates and expressed as proportions (LSM±SEM) of Day-6 cultured embryos (*n*).
Values in the same column with different letters show significant differences: ^{x,y} ($p < 0.01$); ^{a,b} ($p < 0.05$).

6.3.3. HDGF identification in BSA extracts

HDGF protein was identified in commercial albumin preparations used in embryo culture, both embryo-culture tested BSA (Fig. 6.1, lanes 1 and 2) and FAF-BSA (Fig. 6.1, lanes 4 and 5). Western blot revealed an immunoreactive 28-kDa band with the molecular weight predicted for HDGF in albumin samples (BSA, lane 1; FAFBSA, lane 4). An extra band, 62-kDa, was detected in BSA samples, which probably corresponds to HDGF tightly bounded to BSA. The presence of HDGF was not observed within the analyzed PVA samples (Fig. 6.1, lanes 3 and 6).

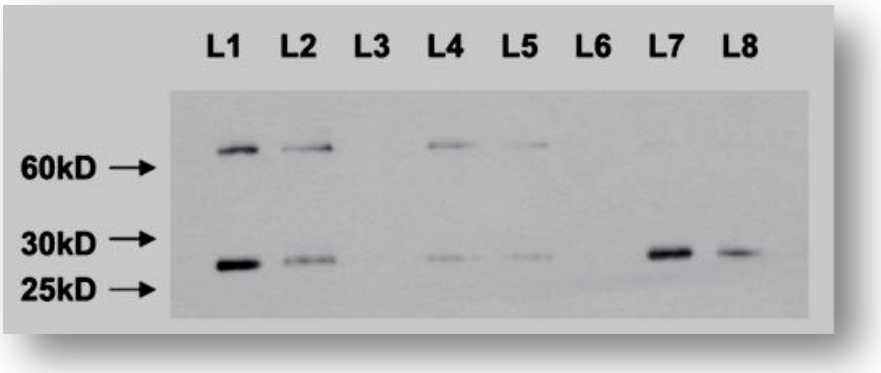

Fig. 6.1. Western blot analysis of HGDF in bovine cold-precipitated serum albumin (BSA) and bovine fatty-acid-free serum albumin (FAF-BSA). Albumin samples were diluted in water (BSA, Lane 1; FAF-BSA, Lane 4) and in Synthetic Oviduct Fluid (SOF) or (BSA, Lane 2; FAF-BSA, Lane 5); lanes 3 and 6 are BSA-free SOF with 0.5 mg mL^{-1} polyvinylalcohol. HGDF was also detected in positive control samples (bovine endometrium, Lane 7 and bovine uterine fluid Lane 8).

6.3.4. Pregnancy and birth rates, and calf measurements, of embryos cultured with rHDGF

Expanded blastocysts produced with or without rHDGF in culture from Day-6 to Day-7 were transferred after vitrification and warming. Results are shown in recipients that were transferred once or twice, and three or four times (Table 6.4). Pregnancy rates as main effects differed between recipients transferred

once twice vs. three-four times within Day-60 pregnancy rate (P < 0.03) and birth rate (P < 0.01) (not shown). However, pregnancy and birth rates did not differ between embryos cultured with and without r-HDGF.

Table 6.4. Pregnancy and calving rates of recipients transferred 1-2 times and 3-4 times with bovine vitrified Day-7 expanded blastocysts individually cultured from Day-6 to Day-7 in 12 µL of synthetic oviduct fluid medium + 0.5 mg mL^{-1} PVA with or without rHDGF (100 ng mL^{-1}).

HDGF	Transfers	Pregnancy rates (%)		Birth (%)
		Day-40	Day-60	
(-)	1-2	20/32 (62)	20/32 (62)	12/24 (50)
(+)	1-2	6/16 (37)	6/16 (37)	6/16 (37)
(-)	3-4	0/3 (0)	0/3 (0)	0/3 (0)
(+)	3-4	2/4 (50)	1/4 (25)	0/4 (0)

Pregnancy and birth rates expressed as % (*n* pregnant/*n* transferred) at each specific endpoint; i.e. some pregnancies are in course).

At birth, calf weight, the other calf measurements, and daily gain weight did not differ between calves from embryos cultured with and without r-HDGF (Table 6.5). Average mother weight at birth did not influence birth weights of embryos cultured with (529 ± 60 Kg) or without (515 ± 42 Kg) r-HDGF (not shown).

Table 6.5. Weight and morphometry of calves born after transfer of bovine vitrified/warmed Day-7 expanded blastocysts cultured individually with or without r-HDGF (100 ng mL^{-1}) from Day-6 to Day-7.

HDGF	*n*	BW (Kg)	HW (cm)	TC (cm)	GL (days)	DG (g day^{-1}
(+)	6	46.4 ± 5.9	80.8 ± 5.9	82.7 ± 3.9	282.2 ± 3.7	164 ± 19
(-)	12	45.6 ± 4.4	78.3 ± 4.2	79.1 ± 2.8	285.9 ± 2.7	159 ± 14

Body weight (BW), height at withers (HW), thorax circumference (TC), gestation length (GL), and average daily gain weight (DG) of the fetus. Differences were non-significant (P > 0.3). Data are LSM±SEM.

6.3.5. Gene expression of HDGF in endometrial cell cultures

In the gene expression study, N = 4 biological replicates from N = 4 animals were validated and used. Endometrial samples analyzed were N = 6 with male embryos, N = 4 with female embryos and N = 4 controls with no embryo. HDGF expression from EC cultured with a male embryo tended to be lower than

control cells with no embryo (P < 0.08; Fig. 6.2). In the presence of a female embryo, EC did not show such tendency.

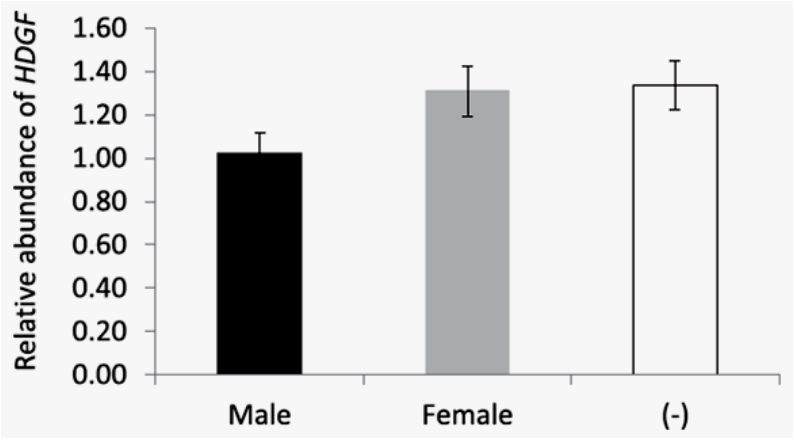

Fig. 6.2. Quantitative real-time PCR analysis of HDGF relative expression in epithelial cells cultured for 48 h with one single male embryo, one single female embryo or no embryos (control) (P < 0.05). (N = 4 biological replicates from N = 4 animals, providing *n* = 6 samples with male embryos, N = 4 with female embryos and N = 4 controls with no embryo).

6.4. Discussion

We identified here new aspects related to HDGF activity in bovine. *In vitro* embryo culture with r-HDGF showed lack of effect in the presence of protein, i.e. the conventional commercial BSA extracts. It is to understand this result that we used WB to analyze two common serum extracts in embryo culture experiments, i.e. BSA Fraction-V obtained by cold-precipitation, and FAF-BSA. We found that both BSAs contained appreciable levels of HDGF, which could be responsible for the lack of effect of exogenously added rHDGF to the culture. The presence of HDGF is not surprising in whole BSA, as this is a blood-borne extract. More interesting is the fact that the procedure for removing fatty acids is respectful with proteins that can be carried by BSA, such as HDGF, suggesting that studies testing GFs should first analyze their presence in the protein extracts used in order to discard confounding effects.

In the absence of protein, however, the effects of rHDGF were obvious, consistent with our previous work [14]. In that study, performed without discrimination of embryonic stages on Day-6, we indicated that HDGF helps to advance the day 6 embryos throughout blastulation. In the present work we refined such finding, so far embryos that started blastulation were remarkably the target for the developmental stimulation exerted by rHDGF, likely together with some embryo termed as morula but that could have showed incipient blastulation (as observed by the numerically higher morula rates). Such effects can be explained as HDGF drives the epithelial to mesenchymal transition (EMT) in cancer and healthy cells, by downregulation of E-cadherin expression and upregulation of vimentin expression [34,35]. Interestingly, in the bovine blastocyst the trophectoderm formation is defined by markers cadherin and vimentin during the EMT [36]; therefore, the blastocyst can be a target for rHDGF.

In this work, the tendency of HDGF to decrease in UF, as analyzed by MRM, may contrast with previous work using 2D-DIGE, whereby HDGF in UF increased in the presence of multiple embryos [14]. However, our results can be in line with the lack of endometrial transcription to HDGF reported in response to the presence of embryos [14]. No correlation between HDGF mRNA and protein levels has also been described in a number of mouse tissues [39], leading to suggest that HDGF undergoes post-transcriptional and/or post-translational regulation. We assume that total HDGF was measured, as the peptide identified with MRM is not susceptible of post-transcriptional modifications. In contrast, unlike in this MRM approach, 2D-DIGE typically excludes other spots with HDGF that can represent post-transcriptional

modifications, as based in different m/w ratio. Such differences could explain our findings, pointing out that post-transcriptional effects can be relevant in the uterus. Noteworthy, embryos drive the differential changes in proteins found in UF acting through the endometrium, apparently without non-physiological reactions [17]. At these early stages, protein of the embryonic masses present in the UF can be considered as negligible (0.15 µg protein/embryo [37]; 7.5 µg protein/50 embryos transferred) in comparison with the total protein recovered from the uterus (4500-5000 µg) [13] and the dilution effect of 3100 µg protein renewal of endometrial origin in the UF per uterine horn in a day [38].

Whether cultured EC are able to react to male embryos by reducing the expression level of HDGF is intriguing. Sexual dimorphism in the early embryo development is mediated by molecular changes in UF in the live uterus [24]. Facultative maternal responses to early embryos could be required for purposes of gender balance or particular sensitivity to GFs among embryos of each sex [40]. Our results of HDGF expression in EC cultures would be not observed if embryos were not sexed (i.e. comparing embryo vs. no embryo). Thus, exposure of early embryos *in vitro* to the maternal cytokine Colony-Stimulating Factor 2 differentially affects growth and interferon tau release from male and female embryos in vivo [41].

Supplementation of embryo CM with 100 ng mL^{-1} rHDGF was compatible with pregnancy and birth of calves with normal weight, gestation length and morphometry. Our study comprises limited numbers of embryo transfers, therefore it is still unknown if the increase in blastocyst production obtained with rHDGF [this work; 14] is accompanied by improved or conserved embryo viability. In terms of ICM and TE cell numbers, the quality of embryos produced

with rHDGF surpasses that of the medium without rHDGF [14], yet it is unclear how such aspects can be related to our embryo transfer results. The effects of rHDGF on Day-7 blastocyst production, particularly acting on the Day-6 early blastocyst, are strictly stage dependent and better observed in individual than group culture [14]. Stage-specific expression of growth factors and receptors has been previously reported in mammalian pre-implantation embryos [42-45]. Therefore, developmentally-enhancing effects of HDGF can be associated with timing of activation of nucleolin, the HDGF receptor gene and protein, during the early bovine embryo development [11]. HDGF lacks a conserved secretory sequence signal present in classically secreted proteins, and its mechanism of extracellular secretion remains to be characterized. Thus, in ovarian cancer cells, HDGF is passively released by necrotic and late apoptotic cells [46]. In the uterus, where intense remodeling and cell detachment takes place, such a way of integrating HDGF in the UF could be feasible.

Perhaps rHDGF can be tested in culture with recombinant BSA as a carrier with properties different from the presumably inert PVA. In addition, as HDGF is a protein with specific glycosaminoglycan-binding residues [47] that serves to promote its internalization by cells [34], supplements as heparin/heparan sulphate or hyaluronan could modulate properties of rHDGF in culture. Adding HDGF to culture medium can improve the performance of cultures of individual embryos, allowing studies for modeling human embryo culture. In addition, cow donors that provide few viable oocytes by aspiration often require individual oocyte culture [48,49]. Otherwise, selective use of rHDGF could be reserved for specific embryonic stages (i.e. perhaps Day-6 early blastocysts, better than Day-6 morulae). Provided that the endometrium could react to the embryonic

sex by changing HDGF gene expression levels, studies dealing with culture of sexed embryos with rHDGF should be accomplished. In this sense, we verified that the sex ratio of Day-7 expanded blastocysts that derive from Day-6 early blastocysts in our culture system is significantly skewed to females [22]. More research is needed to identify precise culture conditions for rHDGF within bovine *in vitro* embryo culture.

In conclusion, the absence of products of animal origin in culture allows sanitary safe conditions. The use of maternal GFs in chemically defined conditions may approximate *in vitro* culture to the uterine environment without using products of animal origin. Species specific recombinant proteins, rarely used in bovine embryo culture, may help in this goal. Similar to other GFs identified in the uterus with a potential role during early development, HDGF acts selectively on specific embryonic stages. In addition, care should be taken with specific requirements derived from the nature of protein used (i.e., HDGF binding) within chemically defined conditions. Supplements used in culture can contain the GF in study, such as BSA with HDGF, thereby making it difficult to analyze in reliable conditions. Ultimately, the use of HDGF is compatible with pregnancy and birth of normal calves.

6.5. References

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7 | DISCUSIÓN GENERAL

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La creciente demanda de proteína de origen animal y el desarrollo de una agricultura de precisión han incrementado notablemente el desarrollo de las tecnologías embrionarias. Las técnicas de producción de embriones *in vitro* han mejorado en gran medida desde su invención. Así, la optimización de sistemas y medios de cultivo ha permitido mejorar la viabilidad de los embriones PIV (Wrenzycki, 2016; Sirard, 2018).

En los dos primeros capítulos de la presente memoria de tesis, el sistema de cultivo *in vitro* implicó el cultivo de zigotos en grupo en medio SOFaaci suplementado con BSA desde día 1 hasta día 6. Más adelante, en el día 6, se seleccionaron mórulas (M) y blastocistos tempranos (EB) de buena calidad, los cuales se cultivaron individualmente en condiciones químicamente definidas reemplazando la BSA por PVA.

Observamos que el cultivo individual sin proteína desde el día 6 aumentó los porcentajes de blastocisto expandido (XB) en día 7 obtenidos a partir de EB de día 6, frente a los obtenidos de M en día 6. Además el cultivo individual durante 24 h en un medio libre de proteína produjo blastocistos expandidos de día 7 con mayores índices de nacimientos después de la vitrificación y la transferencia a receptoras. También se obtuvieron evidencias de que la cinética embrionaria y la vitrificación pueden impactar en los fenotipos al nacimiento, especialmente en hembras. De este modo, después de la vitrificación de embriones, la transferencia de EB-XB a receptoras produjo terneros con menores pesos al parto que cuando se transfirieron M-XB. Sin embargo, en el cultivo individual sin proteína solo el 10% de los embriones alcanzaron el estadio de EB en día 6.

Tratando de aumentar los bajos índices de EB en día 6, en el capítulo tres evaluamos si una concentración mínima de suero era capaz de mejorar los porcentajes de EB en día 6. De esta manera, el cultivo individual sin proteína posterior al día 6 produciría más XB en día 7. El uso de una concentración muy baja (0.1%) de suero en el cultivo hasta día 6 permitió triplicar la producción de EB y obtener así más XB de esta transición. En términos de calidad y viabilidad, los XB de día 7 cultivados con suero hasta el día 6 son comparables a los embriones producidos en SOF-BSA. Finalmente, en el cuarto capítulo se demostró que la concentración de HDGF total en el fluido uterino (FU), tendió a aumentar con la presencia de embriones en el útero. Además, se estableció que la expresión de HDGF fue menor en células endometriales cultivadas con un embrión macho frente a los cultivos de esas células sin embriones. Por último, se constató que la acción de HDGF recombinante (rHDGF), a partir del día 6 en cultivos individuales con o sin proteína, es dependiente del estadío embrionario y que la presencia de BSA puede enmascarar tales efectos.

7.1. Restricción de proteína y desarrollo de embriones

Solo el 10% de los embriones alcanzó el estadio de EB en el Día 6, y los porcentajes de desarrollo de M a XB fueron menores en ausencia de proteína. La ausencia de proteína redujo los porcentajes de blastocistos y la expansión en los días 7 y 8 a partir del estadío de M pero no a partir del estadío de EB en día 6. En bovino, durante el cultivo de embriones *in vitro*, los estadíos posteriores a la compactación requieren más proteína (Thompson, 1998) y aminoácidos (Guerif *y cols.,* 2013) para su desarrollo que durante las primeras

etapas de segmentación. El requerimiento de proteína parece ser estrictamente dependiente del estadío embrionario ya que, en contraste con la mórula, el EB de día 6 produce altos porcentajes de desarrollo y expansión en día 7 con y sin proteína.

Los estudios de cinética en embriones bovinos generalmente se han ocupado de analizar estadíos finales de desarrollo. Sin embargo, raramente se ha considerado el estadio de origen, a pesar de que para seleccionar embriones de viabilidad superior, la evaluación del desarrollo embrionario según patrones específicos se ha propuesto como más adecuada (Gutiérrez-Adán *y cols.,* 2015).

Varios estudios han demostrado que la presencia de suero, a pesar de que puede inhibir las divisiones tempranas, estimula el desarrollo hasta el estadío de blastocisto (Rizos y cols., 2003; Loureiro *y cols.,* 2009; Francis, 2010). Por tanto, con el fin de aumentar los porcentajes de EB se agregaron proporciones muy reducidas de suero desde el día 1 al día 6, antes de la restricción de proteína. A partir de entonces, el estadío de EB, producido con y sin suero en cultivo hasta día 6, dio lugar a mejores rendimientos de XB en día 7. Estos resultados sugieren que el EB requiere menos sustrato que el estadío de M para alcanzar la etapa XB.

La adición de rHDGF en presencia de BSA no mostró efecto alguno sobre el desarrollo de blastocistos; sin embargo, en el cultivo libre de BSA, el rHDGF promovió principalmente el desarrollo de EB en contraste con M. Además, los efectos de rHDGF en la producción de blastocistos de día 7, se evidenciaron principalmente en EB de día 6. Tales efectos son estrictamente dependientes

del estadío y se observan con más claridad en cultivo individual que en cultivo en grupo (Gómez *y cols.,* 2014). El HDGF dirige la transición epitelio-mesenquimal en células cancerígenas y en células sanas mediante la reducción de la expresión de E-cadherina y el aumento de la expresión de vimentina. Curiosamente, en el blastocisto bovino, la formación del TF también está definida por los marcadores de cadherina y vimentina durante la transición epitelio-mesenquimal (Yamakoshi *y cols.,* 2012); por lo tanto, el blastocisto puede ser un blanco para el rHDGF.

Por otro lado, encontramos que tanto la BSA como la BSA libre de ácidos grasos analizadas en el último estudio contenían niveles apreciables de HDGF, lo que podría explicar la falta de efecto del rHDGF exógeno en cultivo. La presencia de HDGF en un extracto del suero como BSA Fracción-V, obtenido por precipitación sin modificación ulterior alguna, es en cierto modo esperable. Sin embargo, sorprende conocer que el procedimiento para eliminar los ácidos grasos de la BSA no ocasiona pérdida de bioactividad de algunas proteínas que la BSA puede transportar, como el HDGF. De esta manera, se debe analizar la presencia de un GF determinado en los extractos de proteína utilizados antes de estudiar sus efectos en cultivo, a fin de descartar posibles efectos indeseados.

Con los estudios que componen esta tesis hemos comprobado que usando cultivo individual sin proteína (condiciones químicamente definidas) es posible realizar análisis no invasivos del medio de cultivo (Gómez *y cols.,* 2016) más precisos y garantizando la trazabilidad de la muestra. Además, el medio químicamente definido permite que otras moléculas de concentración conocida

puedan ser añadidas (por ejemplo rHDGF) al medio de CIV para evaluar sus efectos.

7.2. Morfología y recuento diferencial de células del blastocisto después de la restricción de proteína

El estudio morfológico del blastocisto en día 7 después del cultivo individual con o sin proteína indica que la ausencia de proteínas no provoca diferencias morfológicas notables. Sin embargo, la ausencia de proteína en día 6 redujo el número de células en la MCI de los blastocistos de día 8. La ausencia de proteínas durante todo el cultivo *in vitro* puede tener un impacto negativo sobre el desarrollo embrionario y afectar al número de células en embriones cultivados en grupos (Krisher *y cols.,* 1999; Duque *y cols.,* 2003; Orsi y Leese, 2004). Sin embargo, el menor número de células MCI en embriones cultivados sin proteína contrasta con la superior viabilidad a largo plazo que muestran estos embriones en el útero, a juzgar por el menor número de abortos que se producen.

Aunque un número mínimo de células embrionarias es necesario para establecer una gestación (Iwasaki *y cols.,* 1990), el número óptimo de células y su distribución en el blastocisto no están claros.

En el capítulo tres, los embriones que provenían de cultivos *in vitro* en presencia de concentraciones reducidas de suero presentaron un color más oscuro en su citoplasma cuando se evaluaron con la luz del estereomicroscopio en día 6. Esta característica se mantuvo en parte en los XB de día 7 después

del cultivo individual sin proteína. El número de células totales en blastocistos eclosionados que sobrevivieron a la vitrificación/calentamiento (v/c) no se vio afectado por la presencia de suero o el estadío embrionario en día 6.

7.3. Restricción de proteína y supervivencia a la criopreservación

La ausencia de proteína favoreció la supervivencia *in vitro* de los XB vitrificados en día 7. Todos los embriones v/c se reexpandieron tras 2 h de cultivo, lo que permite eliminar el cultivo previo a la transferencia, una operación costosa y que consume mucho tiempo. Curiosamente, después del cultivo individual sin proteína, ningún blastocisto fresco eclosionó en día 7. En el capítulo tres, el uso de FCS permitió seleccionar más blastocistos expandidos para ser vitrificados en día 7. La presencia de FCS hasta el día 6 no afectó los porcentajes de supervivencia *in vitro* después de la vitrificación de los XB en día 7. También observamos que los XB de día 7 que fueron producidos con FCS tendieron a eclosionar menos al cabo de 24 h. Sin embargo, esto puede interpretarse como un retraso, ya que los porcentajes de eclosión se igualaron en todos los grupos al cabo 48 h.

Sudano *y cols.,* (2012) observaron que la incidencia de apoptosis en células embrionarias muestra una alta correlación con la supervivencia del embrión después de la criopreservación. También se ha demostrado que la suplementación con suero aumenta el número de células embrionarias (Gardner *y cols.,* 2000; Van Langendonckt *y cols.,* 1997), aunque otros estudios documentaron una reducción (Okumu *y cols.,* 2014) del número de células. En

nuestro estudio, observamos que ni la suplementación con FCS ni el estadío embrionario en día 6 (M o EB) aumentaron los porcentajes de apoptosis de blastocistos que eclosionaron después de la v/c. Además, el número total de células en los blastocitos eclosionados que sobrevivieron a la vitrificación no se vió afectado por la suplementación con FCS o el estadío embrionario en día 6.

7.4. Contenido de lípidos intracelulares después de la restricción de proteína

La reducción en el contenido de lípidos y su movilización, mejoran la supervivencia a la criopreservación y disminuyen el índice de apoptosis en embriones bovinos PIV (Ghanem *y cols.,* 2014). La proteína exógena y el estadío embrionario en día 6 afectaron al contenido de lípidos observado en XB de día 7. La suplementación con proteína durante el CIV modificó el contenido de lípidos de XB en día 7 formados a partir de M y EB en día 6. Las células embrionarias en estadío de M contienen más gotas lipídicas que en EB (Sudano *y cols.,* 2016). Sin embargo, las reservas de lípidos se redujeron en EB-XB, independientemente de la presencia o ausencia de proteína.

Con el inicio de la compactación de la M y durante la formación del blastocisto, las demandas de energía, analizadas como consumo de oxígeno y nutrientes, aumentan drásticamente. La capacidad de usar oxígeno en la M comparada con el EB podría estar aún limitada y, por tanto, entrañar una mayor dificultad para movilizar lípidos en ese estadío. En este sentido, Sudano *y cols.,* (2016) describen que la acumulación de lípidos es mayor en estadíos de M frente a

etapas posteriores. La lipogénesis está asociada con los gotas lipídicas más pequeñas, las cuales convergen para formar gotas más grandes mediante diferentes mecanismos (Thiam *y cols.,* 2013). De esta manera, los embriones M-XB cultivados sin proteína mostraron menos gotas pequeñas y totales de lípidos que los embriones M-XB cultivados con proteína, sugiriendo que la eliminación de proteína condujo a una degradación de lípidos más pronunciada en embriones M-XB.

La fracción lipídica presente en el suero puede incorporarse a los embriones en desarrollo (Abe *y cols.,* 2002) y afectar a la calidad de los blastocistos resultantes (Leroy, 2010). En nuestro estudio, la ausencia de FCS en el medio de cultivo redujo la acumulación intracelular de lípidos. La interacción entre el FCS y el estadío embrionario no afectó a la acumulación de lípidos en XB de día 7. Sin embargo, la presencia de 0,1% de FCS en cultivo aumentó la acumulación de lípidos en XB, aunque la supervivencia de los embriones a la v/c y los porcentajes de apoptosis fueron similares a los embriones producidos en BSA.

7.5. Expresión génica tras la restricción de proteína

Los patrones de expresión génica que muestran los embriones frescos desaparecieron en los blastocistos que sobrevivieron a la vitrificación. En los blastocistos frescos, los cambios en la expresión génica a partir de la supresión de proteína se circunscribieron a la degradación de los lípidos y la apoptosis. En nuestro estudio, el incremento en la expresión de *G6PD* en embriones

cultivados sin proteína puede ser reflejo de una capacidad superior de tales embriones para contrarrestar el estrés oxidativo inducido por la criopreservación. Es posible también que esta capacidad pueda asociarse a una mayor viabilidad embrionaria y a un menor porcentaje de abortos espontáneos, tal como hemos observado.

En embriones bovinos, la sobreexpresión de *BAX* está vinculada a embriones de menor calidad (Vandaele *y cols.,* 2008). Por tanto, la expresión reducida de *BAX* en embriones cultivados sin proteína sugiere mayor calidad de estos embriones. La falta de diferencias en la expresión de *GPX1* y *SOD2*, genes asociados a las mitocondrias y codificantes de enzimas antioxidantes, tanto antes como después de la criopreservación, indica ausencia de estrés oxidativo inducido por la supresión de proteína. Por el contrario, encontramos *ATF4* y *DDIT3* sobreexpresados en XB de día 7 que fueron privados de proteína antes de la criopreservación, lo cual indica que el estrés causado por la restricción de proteínas se limita al retículo endoplasmático. Curiosamente, estas diferencias, junto con la mayor expresión de *IGF2R* en embriones frescos, no se observaron en los blastocistos que sobrevivieron a la criopreservación. Por el contrario, la ausencia de proteína (Lim *y cols.,* 2007) y diferentes sistemas de CIV (con suero o BSA) no alteraron la expresión de *IGF2R* con respecto al desarrollo *in vivo* en el oviducto ovino o en la vaca (Lazzari *y cols.,* 2002).

En nuestro estudio encontramos que la sobrerregulación de *IGF2R* en embriones cultivados sin proteína se asemeja más a las condiciones fisiológicas de los embriones en el tracto uterino. Por el contrario, una menor

expresión de *IGF2R* se ha relacionado con el crecimiento excesivo del feto (Young, 2001) y el aborto en clones de ganado bovino (Yang *y cols.,* 2013).

La expresión génica no se evaluó en nuestros experimentos de suplementación con FCS. Sin embargo, en un estudio realizado por Heras *y cols.,* (2016) el transcriptoma de embriones producidos en condiciones libres de suero mostró una mayor semejanza con el transcriptoma de embriones *in vivo*, frente a los embriones cultivados en presencia de suero.

7.6. Restricción de proteína y dimorfismo sexual

El dimorfismo sexual durante el desarrollo embrionario temprano *in vivo* se evidencia en los cambios moleculares en el fluido del útero (Gómez *y cols.,* 2014). Las mórulas y los blastocistos difieren en el número de genes expresados ligados al sexo y estas diferencias aumentan a medida que el embrión se desarrolla en la etapa de blastocisto (Denicol *y cols.,* 2015). Sin embargo en nuestro estudio no encontramos cambios en la proporción esperada de embriones macho/hembra durante la transición de M-XB. Sin embargo, observamos porcentajes más elevados de embriones hembras dentro de las transiciones de EB-XB en comparación con M-XB. En bovino, cuando no se considera un patrón de desarrollo previo, aparece un mayor número de machos que de hembras entre los blastocistos que se desarrollan más rápido (Gutiérrez-Adán *y cols.,* 1996, Larson *y cols.,* 2001, Kimura *y cols.,* 2005). Sin embargo, un cultivo individual de embriones por 24 h en SOFaaci con proteína no alteró la proporción total de sexos en los blastocistos (Muñoz *y*

cols., 2014a, 2014b). Las discrepancias podrían explicarse por la reducción de las proporciones de EB entre los embriones de día 6 (≈10%), ya que la proporción de sexos se ve influida solo en una baja proporción de los embriones analizados, de tal manera que el efecto puede ser inadvertido.

Curiosamente, los efectos sobre la proporción del sexo embrionario se asociaron claramente al estadío y no a la proteína, a pesar de que los embriones masculinos y femeninos difieren en el metabolismo de aminoácidos (Sturmey *y cols.,* 2010) y en la regulación de proteínas que inducen en el fluido uterino (Gómez *y cols.,* 2013). En nuestro sistema de cultivo verificamos que la mayor proporción de XB de día 7 que derivan de EB en día 6 está significativamente sesgada hacia el sexo hembra. No obstante, Heras *y cols.,* (2016) describen que los embriones bovino PIV de sexo hembra parecen ser los menos afectados por las condiciones subóptimas de CIV (por ejemplo presencia de suero), siendo los embriones macho PIV los que muestran hasta tres veces más genes expresados diferencialmente comparados con embriones *in vivo* y con embriones *in vitro* hembra.

Los cultivos *in vitro* de células endometriales tendieron a reducir su nivel de expresión de HDGF ante la presencia de un embrión macho. La respuesta a la presencia del sexo del embrión temprano podría ser necesaria para adaptar el ambiente uterino a las distintas necesidades de los embriones macho o hembra, o bien podría obedecer a la particular regulación de determinados GFs entre embriones de cada sexo (Hansen *y cols.,* 2016). Nuestros resultados de expresión de HDGF en cultivos de células endometriales no se observarían si

no se hubiese considerado el sexo del embrión cocultivado (es decir, si comparamos solamente presencia o ausencia de embrión).

7.7. Gestación y resultados perinatales

En bovinos, un objetivo importante de la producción de embriones *in vitro* es mejorar los porcentajes de XB en día 7. En general, los XB de día 7 muestran índices de gestación más elevados que los embriones del día 8 y que estadíos embrionarios anteriores (Randi *y cols.,* 2016; Kubisch *y cols.,* 2004; Van Wagtendonk- de Leeuw *y cols.,* 1998).

Los experimentos de supervivencia *in vitro* no han logrado predecir bien las diferencias en el porcentaje de nacimientos y abortos espontáneos ocasionados por embriones PIV. La restricción de proteína en el CIV redujo los porcentajes de aborto tardío, sugiriendo que el paso por un medio de cultivo sin proteína es beneficioso para lograr gestaciones a término. De hecho, en bovino, los blastocistos que se encuentran en el útero actúan reduciendo el volumen de fluido uterino secretado, medido como proteína total recuperable; y esta reducción se observa específicamente en la concentración de albúmina (Muñoz *y cols.,* 2012). Como el paso por el útero es beneficioso para la viabilidad embrionaria y la supervivencia a la criopreservación (Rizos *y cols.,* 2002; Lonergan *y cols.,* 2004; Havlicek *y cols.,* 2010), los efectos beneficiosos a largo plazo que observamos después de un corto periodo de supresión proteica durante el desarrollo tardío del blastocisto *in vitro* podrían ser equivalentes a los cambios fisiológicos que ocurren en el útero *in vivo*. Además,

ese mismo efecto de reducción de proteína total inducido por un embrión macho también se ha observado en cultivos de células endometriales epiteliales (Gómez *y cols.,* 2018b).

Los terneros nacidos después de la transferencia de EB-XB vitrificados en día 7 pesaron menos al nacimiento que los terneros derivados de M-XB de día 7. En bovinos, un peso elevado al nacimiento aumenta los riesgos de distocia, muerte fetal, mortalidad neonatal y daño a la madre (Van Wagtendonk-de Leeuw *y cols.,* 1998; Bonilla *y cols.,* 2014). Por lo tanto, la transferencia de EB-XB vitrificados puede mejorar la facilidad de parto y el bienestar feto-materno. En los estudios con embriones vitrificados, la falta de proteína tendió a incrementar los porcentajes de nacimientos mediante la reducción del aborto espontáneo, lo que disminuye los perjuicios económicos, productos y de bienestar animal derivados de las pérdidas de gestaciones tardías. Curiosamente, la eliminación de proteínas durante la formación del blastocisto no afectó el peso al nacimiento, la duración de la gestación o la morfología de los terneros.

En bovino, el peso al nacimiento aumenta después de la transferencia de embriones cultivados *in vitro* en medio SOF con suero o con 16 g L^{-1} de BSA (Lazzari *y cols.,* 2002), concentraciones ambas superiores a las utilizadas en los experimentos de esta tesis. En el estudio de Lazzari *y cols.,* (2002) más del 50% de los terneros nacieron con 50 kg de peso, en contraste con nuestro estudio en donde obtuvimos una menor proporción de terneros con pesos elevados, aunque el número de casos no es aún elevado.

Los embriones bovinos CIV con FCS (0.1%) hasta el día 6 transferidos produjeron índices de gestación comparables a los producidos con embriones cultivados sin suero. Nuestros resultados preliminares sugieren que una baja concentración de FCS antes del cultivo individual sin proteína produce embriones viables a partir de varios estadíos embrionarios. Sin embargo, los efectos a largo plazo de la suplementación con suero asociada con el peso al nacimiento y el fenotipo de los terneros deben evaluarse en estudios futuros.

La suplementación de los medios de cultivo de embriones *in vitro* con 100 ng mL^{-1} de rHDGF fue compatible con gestaciones y el nacimiento de terneros con pesos, duración de la gestación y morfometría normales. Nuestro estudio comprende un número limitado de transferencias de embriones; por lo tanto, aún se desconoce si el aumento en la producción de blastocitos obtenidos con rHDGF conserva o mejora la viabilidad embrionaria. En cultivos en grupo de mórulas en medio libre de proteína y con PVA, rHDGF inhibió el desarrollo de blastocistos y no afectó al número de células cuando las mórulas eran tempranas (día 5), sin embargo, mejoró el desarrollo de blastocistos y aumentó el recuento celular cuando las mórulas eran compactas (día 6) (Gómez *y cols.,* 2014).

El cultivo individual proporciona acceso rápido y directo a los medios de cultivo. De esta manera es posible realizar estudios de biomarcadores para determinar, de manera no invasiva, la viabilidad de la gestación (Muñoz *y cols.,* 2014a, 2014b), el metabolismo de aminoácidos (Sturmey *y cols.,* 2010) y diagnosticar el sexo del embrión (Muñoz *y cols.,* 2014c; Gómez *y cols.,* 2016; 2018b). Además, la restricción de proteína en cultivo individual es potencialmente

compatible con cualquier otro sistema de cultivo hasta día 6, proporcionando un medio homogéneo y repetible entre laboratorios. Los usuarios que deseen mejorar la supervivencia después de la criopreservación pueden asignar embriones a un medio de cultivo libre de proteína cuando no haya receptoras disponibles o cuando exista un excedente de embriones.

7.8. Referencias

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8 | CONCLUSIONES

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Las conclusiones del presente trabajo de tesis son:

- La ausencia de proteína durante el CI entre D6-D7 produce menos blastocistos expandidos, pero mejora su viabilidad a largo plazo después de la criopreservación.
- Una baja concentración de FCS es suficiente para mejorar los porcentajes de blastocisto temprano en día 6, los cuales generan a su vez más blastocistos expandidos que las mórulas después de un día de cultivo individual sin proteína.
- La calidad de los blastocistos expandidos producidos con muy baja concentración de FCS es similar a los blastocistos producidos con BSA en términos de apoptosis, contenido de lípidos e índices de gestación.
- La cinética del embrión y la vitrificación condicionan los fenotipos al nacimiento. El peso de los terneros aumenta al transferir blastocistos vitrificados que provienen de mórula, un efecto que es más acentuado en las hembras. Las alteraciones están asociadas a la proteína exógena y la movilización de las reservas de lípidos.
- El uso de GFs de origen materno, producidos mediante síntesis recombinante, puede aproximar el cultivo *in vitro* en condiciones químicamente definidas al ambiente uterino.
- El HDGF actúa selectivamente en etapas embrionarias específicas durante el desarrollo temprano, al igual que otros GFs identificados en el útero bovino.
- El uso de rHDGF en cultivo de embriones *in vitro* es compatible con la gestación y el nacimiento de terneros normales.