GOBIERNO DE CHILE
FUNDACIÓN PARA LA
INNOVACIÓN AGRARIA

PROGRAMAS DE
FORMACIÓN y PROMOCIÓN PARA LA INNOVACIÓN

INSTRUCTIVO ELABORACIÓN INFORME TÉCNICO Y DE DIFUSIÓN

Apoyo a la participación en actividades de formación

AÑO 2004

GOBIERNO DE CHILE
FUNDACIÓN PARA LA INNOVACIÓN AGRARIA

INSTRUCTIVO PARA LA PREPARACION DEL INFORME TÉCNICO Y DE DIFUSIÓN

1. OBJETIVO

El objetivo de este informe es el de describir, analizar y evaluar la forma en que se desarrollo la propuesta, tanto desde el punto de vista técnico, como de su gestión administrativa y de las actividades de difusión realizadas. Específicamente, en este informe se deberán describir las visitas y tecnologías conocidas durante la Actividad de Formación, y junto con eso también se deberá contemplar un análisis y reflexión respecto a los conocimientos adquiridos en la actividad y su aplicabilidad concreta en el país o en lugar de origen del participante, incluyendo los desafíos o limitantes que se presentan para su incorporación.

Adjunto al informe se deberá entregar una copia de todo el material o documentación recopilado durante la Actividad de Formación, incluyendo copia del material audiovisual.

El informe deberá adicionalmente describir las actividades de difusión realizadas, de acuerdo con el programa de difusión comprometido en su propuesta, adjuntando el material y documentación utilizada y entregada a los asistentes en dichas actividades.

2. PLAZOS Y ENTREGA DE INFORMES

Luego de terminada la actividad de Formación y del regreso del participante, éste y/o la Entidad Patrocinante tienen un plazo máximo de 2 meses para realizar las actividades de difusión comprometidas en la propuesta. Después de realizada la última actividad de difusión comprometida, disponen de un plazo máximo de 15 días para la entrega a FIA del **Informe Técnico y de Difusión.**

Estos plazos están especificados en el contrato de ejecución respectivo y en la eventualidad de que exista un imprevisto que no le permita al participante y/o Entidad Patrocinante cumplir con dichos plazos, éstos deberán justificar y solicitar por escrito a la Dirección Ejecutiva de FIA la posibilidad de prorrogar los plazos estipulados, los cuales se autorizarán en la medida que existan una razón clara y justificada.

En la eventualidad de que los compromisos antes señalados no se cumplan, se procederá a ejecutar la garantía respectiva y el participante quedará imposibilitado de participar en nuevas iniciativas apoyadas por los diferentes Programas e instrumentos de financiamiento de FIA.

3. PROCEDIMIENTO

Los informes deben ser presentados, preferentemente en disquet o disco compacto y obligatoriamente en papel (tres copias) de acuerdo a los formatos establecidos por FIA, en la fecha indicada como plazo de entrega en el contrato firmado con el participante y/o Entidad Patrocinante. Los formatos de dichos informes (impresos y en disquet) son entregados por FIA al postulante o participante de la propuesta a través de este instructivo.

Los informes deberán ser dirigidos a las oficinas de FIA ubicadas en Avenida Santa María 2120, Providencia, Santiago, y podrán entregarse personalmente en dichas oficinas en horario hábil o enviarse por correo a domicilio en forma oportuna para que llegue en el plazo establecido.

El FIA revisará los informes y dentro de los 45 días hábiles siguientes a la fecha de recepción (plazo máximo) enviará una carta al responsable de la propuesta o participante, informando su aceptación o no aprobación. En caso de no aprobarse el informe FIA comunicará en detalle las razones de dicha decisión. El responsable deberá corregir los reparos u observaciones que motivaron el rechazo, dentro del plazo determinado por el FIA.

4. CONTENIDO Y FORMATO

La información presentada en el informe de avance técnico y de difusión debe ser presentada en un lenguaje claro y estar directamente vinculada a la información presentada en el informe financiero, siendo totalmente consistente con ella.

El informe debe incluir o adjuntar los cuadros, gráficos, fotografías y diapositivas, publicaciones, material de difusión, material audiovisual y otros materiales que apoyen o complementen la información y análisis presentados en el texto central.

El informe de avance técnico y de difusión debe incluir a lo menos información sobre todos y cada uno de los puntos mencionados a continuación, y siguiendo en lo posible el orden indicado.

Es importante contar con toda la información que se solicita, como por ejemplo, los antecedentes de los participantes en las actividades, información relevante para FIA. El envío de la información incompleta puede ser motivo de no aprobación de este informe.

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CONTENIDO DEL INFORME TÉCNICO Y DE DIFUSIÓN

Fecha de entrega del Informe
Septiembre 30 de 2005 de 2005
Nombre del coordinador de la ejecución
Marco Berland Olea
Firma del Coordinador de la Ejecución

1. ANTECEDENTES GENERALES DE LA PROPUESTA
Nombre de la propuesta
ENTRENAMIENTO EN PRODUCCIÓN DE EMBRIONES BOVINOS IN VITRO
Código
FIA-FP-V-2004-1-P-042
Postulante o Postulantes
MARCO ANTONIO BERLAND OLEA
Entidad Patrocinante o Responsable
Escuela Medicina Veterinaria, UNIVERSIDAD CATÓLICA DE TEMUCO
Lugar de Formación (País, Región, Ciudad, Localidad)
Copenhagen, Dinamarca y DUBLIN, IRLANDA
Tipo o Modalidad de Formación (curso, pasantía, seminario, entre otros)
ENTRENAMIENTO
Fecha de realización (Inicio y término)
7 DE ENERO AL 15 DE FEBRERO DE 2005

2. ALCANCES Y LOGROS DE LA PROPUESTA

Justificación y objetivos planteados inicialmente en la propuesta

2.1.- Objetivo General

Aplicar metodologías modernas y efectivas de cultivo que permitan eficientizar la tecnología de producción de embriones bovinos in vitro, como una herramienta para el mejoramiento genético animal.

2.2.- Objetivos Específicos

- Conocer y manejar sistemas de cultivo en base a medios semidefinidos y condiciones de atmósfera controlada de oxígeno (sistema de cultivo SOF.)

- Conocer y comparar los procedimientos de obtención de embriones a partir de hembras vivas.

- Manjar metodologías celulares y de cultivo para evaluar los procedimientos de producción de embriones.

- Conocer el avance y perspectivas actuales de las biotecnologías en embriones y su aplicación en los sistemas productivos.

- Establecer contactos y relaciones para impulsar el desarrollo de actividades de colaboración en investigación y aplicación de biotecnologías en embriones

Justificación

Formo parte de un grupo que por más de 10 años ha estado trabajando en el tema de fecundación de embriones bovinos y también Camélidos Sudamericanos. La investigación en el campo de la Biotecnología Reproductiva, en especial en lo que respecta al mejoramiento genético, a partir de la implementación de tecnologías eficientes y efectivas en la producción de embriones in vitro, ha tomado especial relevancia en estos últimos años. Lo anterior considerando sus aplicaciones efectivas y potenciales en el mejoramiento genético animal y más recientemente en los procedimientos para la obtención de bovinos transgénicos como bioreactores.

En este contexto, nuestro grupo esta desarrollando desde al año 2002 un proyecto FIA de Biotecnología en embriones bovinos (BIOT-01-P08), que ha buscado el desarrollar y evaluar la aplicación de la producción de embriones bovinos como una herramienta para el mejoramiento de la masa ganadera. Para lo cual hemos trabajado con predios de crianza de ganado de la Región. Entre los principales resultados que hemos podido obtener y que ya considerábamos como potenciales riesgos de éxito, se constata que uno de los aspectos críticos para la eficiencia de esta tecnología son las condiciones y metodologías de cultivo de los embriones, que marcan fuertemente los resultados que se pueden obtener. Esto no es extraño ya que la investigación de punta en este aspecto ha demostrado e implementado metodologías más efectivas en este ámbito.

Las nuevas metodologías para el cultivo de embriones van asociadas principalmente a la obtención de mayor número y mejor calidad de los embriones, lo cual se puede evaluar y que es factor determinante en las potenciales preñeces que se obtengan. El conocer y manejar estas nuevas metodologías nos permitirá eficientizar las aplicaciones prácticas y mejoras las proyecciones de nuestro trabajo en este campo. Uno de los objetivos del proyecto que estamos desarrollando es que una vez finalizado la aplicación de estas tecnología se proyecte en el tiempo y sea una herramienta para el mejoramiento genético, que cada día toma mayor importancia en nuestro país como ya lo ha hecho en el mundo desarrollado.

En conjunto con lo anterior nuestro grupo, en conjunto con la Unidad de Biotecnología Animal de INIA CARILLANCA, ha obtenido un proyecto FONDEF para la generación de bovinos transgénicos como Bioreactores (FONDEF D03I1074). Parte de los fondos obtenidos para nuestra unidad nos permitirán adquirir moderno equipamiento para el cultivo de embriones con condiciones mejoradas, y que corresponde a una parte fundamental de los conocimientos prácticos que podría adquirir en el entrenamiento para el cual estoy enviado esta propuesta. Estas condiciones nos permitirán abordar en mejores condiciones el desafío del proyecto en transgénesis y potenciar la producción de embriones in vitro para su aplicación real en el campo productivo.

El grupo de trabajo en donde se desarrollaría mi entrenamiento, esta encabezado, en los principales aspectos de mi interés, por el Dr. Pat Lonergan, quienes son actualmente uno de los grupo más connotados en al ámbito de biotecnologías en embriones de especies productivas. Otro aspecto fundamental es que tengo la posibilidad de realizar un entrenamiento sin costo, hecho que no se da en la mayoría de los centros que son referentes mundiales en el tema.

Finalmente, debo señalar que mi interés y del grupo de trabajo es de continuar trabajando en forma aplicada y en investigación para contribuir efectivamente al desarrollo de estas biotecnología en nuestro país.

Además y tomando en cuenta la eventualidad de encontrarme potencialmente en esa fecha cerca del lugar donde se realizará la Conferencia Anual de la International Embryo Transfer Society, considero de manera relacionada pero a la vez independiente la posibilidad de, con costos sustancialmente menores, de asistir a dicho evento, donde se presentarán trabajos y conferencias por parte de científicos del más alto nivel mundial, en el tema relacionado a nuestro trabajo y a los tópicos del entrenamiento. Por lo anterior incluyo programas y costos para participar en esta actividad. Quiero recalcar que al calcular los costos de asistir a este evento ellos son menores si consideramos que estaré muy cerca de Irlanda donde realizaría mi entrenamiento.

Mi entrenamiento finalizaría el 15 de febrero y esto por diferentes razones. En Europa el feriado de fin de año se extiende hasta el 5 de enero y luego el Profesional encargado del entrenamiento estará ausente entre el 8 y 13 del mismo mes ya que es un connotado científico que participa en las actividades de Congreso mencionado que se realizará en Dinamarca. También estamos en estación opuesta con Europa y según los contactos realizados se hace imposible realizar y finalizar el entrenamiento antes del 28 de enero. Por mi parte es imposible que sea antes ya que tengo que cumplir con mis labores académicas y de finalización del proyecto FIA.

Finalmente y considerando todas las variables que incluyen además la preparación de las actividades para mi entrenamiento, acordamos con el Dr. Pat Lonergan postular la posibilidad de que el entrenamiento termine el 15 de febrero.

Objetivos alcanzados tras la realización de la propuesta

Todos los objetivos que se plantearon en la propuesta fueron alcanzados durante el transcurso del entrenamiento y asistencia al Congreso Mundial de la IETS.

Debo recordar que la propuesta final aprobada consideró en primer término la asistencia al congreso mundial anual de la Internacional Embryo Tranfer Society (IETS), el cual se desarrolló en la ciudad de COPENHAGEN, en DINAMARCA.

La asistencia a este evento me permitió conocer y constatar el avance e importancia mundial de las temáticas asociadas a la Biotecnología en embriones, especialmente en el tema de bovinos. Este evento es uno de los que considero debería estar en la lista de Congresos permanentes para los cuales FIA aporta financiamiento de asistencia. Se realiza todos los años generalmente en un país del mundo desarrollado.

Uno de los logros importantes que pude establecer, fue un vínculo con perspectivas de trabajo en investigación y también amplias posibilidades de realizar estudios de Doctorado en Irlanda. Lo anterior ahora pasa por conseguir el financiamiento para que estos se concrete. Lamentablemente el costo de vida y estudios en EUROPA es muy alto para nuestra realidad, sin embargo hay posibilidades que se deben trabajar con tiempo.

Resultados e impactos esperados inicialmente en la propuesta

1.- La aplicación de técnicas modernas para la obtención de embriones bovinos nos permitirá desarrollar de manera más eficiente la producción de embriones para mejoramiento genético. Su impacto se debería dar en el mediano plazo a través de servicios que se implementarán destinados a la posibilidad de que los ganaderos de la IX Región y otras tengan la posibilidad real de aplicar estas tecnologías en sus sistemas productivos. Este hecho ya se ve con gran factibilidad ya que diversos empresarios del rubro nos han solicitado asesoría para evaluar la factibilidad técnica de estos procedimientos.
2.- Un segundo impacto importante en el mediano plazo será el afianzar vínculos con una de las unidades más importantes de trabajo en este ámbito a nivel mundial. La idea es establecer el contacto y comenzar a trabajar en forma colaborativa con ellos.

3.- Los aspectos a abordar en mi entrenamiento también ayudarán en el corto plazo a mejorar las condiciones de trabajo con embriones que es uno de los elementos bases en el trabajo de nuestro proyecto recientemente aprobado por FONDEF. Lamentablemente y debido a una mala evaluación técnica, FONDEF nos recortó mucho los fondos solicitados y no pudimos obtener dineros para realizar perfeccionamiento en este ámbito.

4.- Por último, la actualización en las temáticas propias es muy importante en mi caso ya que trabajo en la formación de futuros médicos veterinarios

Resultados alcanzados

Describir si se lograron adquirir los conocimientos, experiencias, alianzas u otros resultados que se esperaban alcanzar a través de la participación en la actividad de formación y del desarrollo de las actividades de difusión. Si hay resultados que no se alcanzaron total o parcialmente, indicar las razones que a juicio del participante explican dicha situación.

Durante la estadía de formación, especialmente en el entrenamiento, creo que se lograron claramente los conocimientos, experiencias contactos y vínculos para alianzas estratégicas de trabajo, en el mediano y largo plazo. En lo fundamental se dió todo como lo esperaba ya que la visita al Laboratorio de Fecundación in Vitro y Estación Experimental en Lyon, del University Collage Dublín, se hizo con la debida programación y previo a aceptación de una propuesta clara y contactos previos que hice con el profesor encargado Dr. Patrick Lonergan. Por otra parte el aprendizaje se facilitó por la excelente disposición y acogida que me brindó el Dr. Lonergan, quién además es una persona muy agradable y reconocida mundialmente en el área. Tuve la oportunidad de participar y asistir a todas las actividades de investigación y difusión que realizan en la Unidad, especialmente los estudiantes de postgrado. Me permitió compartir experiencias, comparar con lo que venimos haciendo en nuestro grupo de trabajo etc.

La actividad de difusión en lo concreto se desarrollo como lo esperaba. Mi idea siempre fue el hacer una actividad que dejara mayores conocimientos que una simple explicación teórica. En lo puntual, este tipo de trabajo que es eminentemente práctico requiere de contextualizar ciertos conocimientos, que en lo teórico están disponibles a través de diversas fuentes de información, sin embargo la clave del aprender en este caso se da en la experiencia práctica. En este sentido, una actividad de este tipo no es de carácter masivo y para que tenga un mayor aprovechamiento debe estar focalizada a ciertas personas con intereses claros y conocimientos básicos previos en el tema. Todo lo anterior demandó una actividad de difusión fundamentalmente práctica de larga duración que implicó bastante trabajo pero al final muy gratificante en la respuesta de los participantes. Finalmente es muy importante señalar que la concreción de la actividad de difusión estuvo determinada por dos factores directa e indirectamente vinculados al quehacer propio del trabajo en la Universidad. En primer término los acontecimientos asociados a las protestas estudiantiles por sus demandas, que mantuvieron las instalaciones Universitarias paralizadas por un tiempo prolongado y por otro la demora en el montaje y puesta en marcha de los nuevos equipamientos adquiridos en nuestra unidad y que son base para el montaje de las experiencias prácticas programadas en la difusión.

Aunque no formaba parte de la propuesta inicial aprobada por el FIA, cuando se me otorgó el financiamiento y en la carta de aprobación, se me encargó el tratar de concretar algún tipo de vínculo con el National Food Center con sede en Dublín, particularmente con su entonces director Mr. Declan Troy, quien había asistido como invitado de FIA a participar en un seminario sobre el tema de la carne, a mediados del 2003. Bueno, en mi estadía hice el esfuerzo de entrevistarme con el Sr. Troy, lo que no se logró debido principalmente a imposibilidad de coordinación por mi ubicación y las actividades propias del Sr. Troy en su trabajo, lo cual además coincidió con un

periodo de vacaciones que el tuvo en parte del tiempo que permanecí en Dublín. En todo caso pude escribirle una nota donde le explicaba la situación, además de conversar con su secretaria personal. Estando en Dublín, traté de establecer contacto con alguna persona que hiciera de intermediario en el intento de establecer algún vínculo de trabajo concreto, lo que no logré, debido a las fechas en las que se realizó mi estadía en Irlanda. Dentro de las personas que me parecián más idóneas para estos fines, me contacté con el Dr. Adrián Catrileo, investigador de INIA CARILLANCA y quien trabaja en el área de carne, especialmente en el tema de producción. Resumiendo, a mi regreso a Chile, sostuve algunas conversaciones con el Dr. Catrileo quien se ha mostrado muy interesado en la posibilidad de establecer algún vínculo a través del Sr. Troy con el National Food Center de Irlanda, especialmente ahora que él forma parte de un grupo importante de personas e instituciones que están postulando un proyecto para crear un consorcio de la carne y la idea que conversamos al respecto es que en el marco de este consorcio puedan establecer un vínculo concreto de colaboración con el centro en Irlanda. El Dr. Catrileo ya le ha escrito a Mr Troy, haciendo referencia a mis contactos con él y los intereses en establecer el vínculo.

He tomado el encargo que me hizo FIA de tratar de ayudar a concretar este tipo de vínculo, en el sentido de que la única forma de que se concrete algo es con el compromiso e interés de una persona vinculada a una institución que este realmente trabajando el ámbito de interés particular. El contacto ya está hecho y he pedido al Dr, Catrileo que me tenga al tanto de las conversaciones que se den en este contexto.

Resultados adicionales

Describir los resultados obtenidos que no estaban contemplados inicialmente como por ejemplo: formación de una organización, incorporación de alguna tecnología, desarrollo de un proyecto, firma de un convenio, entre otros posibles.

1) En lo práctico, tuve la oportunidad de ver y participar de la aplicación práctica de la tecnología de transferencia de embriones quirúrgica interespecífica. Esta tecnología consiste básicamente, para el caso del bovino, en transferir embriones bovinos a oviducto de oveja con el fin de realizar cultivo in vivo. Esto permite evaluar y controlar los procedimientos in Vitro, de tal forma de saber si las condiciones de trabajo en laboratorio son las adecuadas, además de ser una importante herramienta para potenciales aplicaciones prácticas, especialmente en la posible congelación de embriones. También pude ver y valorar la aplicación de tecnologías asociadas a la expresión génica en embriones, tecnología que se está utilizando con fuerza en programas de investigación básica y aplicada.

2) El Dr. Lonergan quedó muy interesado y dejó las puertas abiertas para el desarrollo de investigaciones conjuntas así como la posibilidad de trabajos en el ámbito de los Camélidos Sudamericanos, especies de gran interés biológico y productivo con las cuales no ha tenido posibilidades de trabajar y con las que nuestro grupo si lo ha hecho por varios años y con importantes hallazgos

3) Por Ultimo, también tuvimos conversaciones sobre la posibilidad de realizar en Chile algún Seminario internacional a lo cual se mostró abiertamente dispuesto así como

también en el colaborar en los emprendimientos asociados a la creación de algún programa de postgrado en el área, que es uno de nuestros objetivos como grupo a mediano plazo.

Uno de los elementos claves para concretar y aprovechar las oportunidades que se abrieron con mi visita es el contar con mayor tiempo y la participación de otros investigadores con los que pueda canalizar el trabajo que se requiere para todos estos efectos. A comienzos del próximo año volverá a nuestra unidad un colega que se encuentra realizando su programa de doctorado en Canadá. Varios de los planes que pretendemos canalizar pasan por contar con su participación ya que la actividad académica es muy absorbente.

Aplicabilidad

Explicar la situación actual del rubro y/o temática en Chile (región), compararla con las tendencias y perspectivas en el país (región) visitado o donde se ha desarrollado la actividad y explicar la posible incorporación de los conocimientos adquiridos, en el corto, mediano o largo plazo, los procesos de adaptación necesarios, las zonas potenciales y los apoyos tanto técnicos como financieros necesarios para hacer posible su incorporación en nuestro país (región). Para ello se debe tener presente la realidad en la cual se desenvuelven los participantes.

La aplicabilidad de lo aprendido es claramente factible y de hecho ya hay algunos ejemplos, aunque aislados, en algunas regiones de nuestro país. En lo particular la utilización de tecnología asociadas a mejoramiento genético animal, área de aplicación más específica relacionada a los temas de perfeccionamiento abordados en el entrenamiento, es factible en nuestro país y su utilización depende especialmente de que los productores estén dispuestos a invertir concientes de los beneficios que trae la utilización de estas tecnologías. En términos generales, en Irlanda por muchos años se ha venido utilizando las tecnologías de embriones in Vitro y los aspectos nuevos que han mostrado mejoras en los rendimientos, están siendo aplicados en forma paulatina, esto porque si bien se ha avanzado aún hay varios aspectos que son materia de investigación.

En el sentido general de la aplicabilidad de estas tecnologías, su potencial ha sido demostrado y ampliamente difundida, al menos en la IX y X regiones, en el marco del proyecto FIA recientemente finalizado por nuestro grupo. Al menos hoy la mayor parte de los productores y profesionales del área están concientes y con el conocimiento para considerar la aplicación de estas tecnologías en sus sistema productivos. Lo anterior debe necesariamente asociarse a la rentabilidad de los negocios que desarrollen los productores, hecho que igualmente parece poco a poco mostrar cambios importantes.

Por último, el conocimiento y manejo de estas tecnologías nos permite como formadores de médicos veterinarios, el incorporar estos conocimientos en los cursos regulares del área de reproducción así como de forma más especializada en los trabajos de tesis de los mismos. En este sentido ya se nos ha solicitado la recomendación de profesionales recientemente titulados para ser

contratados en una empresa cuyo objetivo principal es el desarrollar tecnologías afines.

Finalmente, es claro que la aplicación práctica directa de las metodologías modernas para producir embriones in Vitro, requerirá de nuestra parte una evaluación consistente y clara antes de ponerla a disposición de los interesados en su aplicación concreta.

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Detección de nuevas oportunidades y aspectos que quedan por abordar

Señalar aquellas iniciativas que surgen como vías para realizar un aporte futuro para el rubro y/o temática en el marco de los objetivos iniciales de la propuesta, como por ejemplo la posibilidad de realizar nuevas actividades.

Indicar además, en función de los resultados obtenidos, los aspectos y vacíos tecnológicos que aún quedan por abordar para ampliar el desarrollo del rubro y/o temática.

Una de las formas de asociar los conocimientos adquiridos y desarrollados en el marco de las biotecnologías reproductivas, de lo cual forma parte el entrenamiento realizado, es buscar los canales más efectivos para el logro de una asociación entre Universidad-Investigadores y productores, en unidades del tipo de "Núcleos Genéticos", las cuales pueden claramente tener un potencial en la aplicación de estas tecnologías en forma local y en ampliar las posibilidades de negocios en el ámbito de las exportaciones. De hecho en conversaciones sostenidas con representantes de empresas internacionales en el rubro, manifestaron un gran interés en poder importar material genético desde Chile, ya sea en forma de semen o embriones bovinos. Todo lo anterior gracias a la imagen país y a las condiciones sanitarias adecuadas para la ganadería. Creo que aquí hay un enrome potencial no aprovechado, estamos perdiendo una gran oportunidad con mayor valor agregado. Creo que los ganaderos nacionales son muy tradicionales y trabajan de manera aislada del mundo de la generación del conocimiento. También se ve un trabajo aislado por pequeños grupos de tratan de imponer sus líneas de emprendimiento, especialmente en lo referido a razas y formas de producción. En Irlanda tuve la oportunidad de ver varios ejemplos de una gran organización liderada por los ganaderos y el gobierno. La sociedad de productores ganaderos de Irlanda es muy organizada, poseen un semanario de divulgación de todas las actividades, una página web y todo coordinado con una oficina gubernamental, que ayuda al desarrollo de la sociedad de productores. En la realidad de nuestro país se ve a muchos ganaderos fragmentados en su organización y constantemente descontentos y contraponiendose a los lineamientos del las entidades gubernamentales.

3. ASPECTOS RELACIONADOS CON LA ORGANIZACIÓN Y EJECUCIÓN DE LA PROPUESTA

Programa de actividades

Fecha	Actividad	Objetivo	Lugar
9-11 enero/ 2005	The 31st Annual Conference of internacional Embryo transfer Society	- Actualización de conocimientos en el campo de biotecnología animal	Falconer Center Copenhagen DINAMARCA

12 Enero 2005	Agricultural and Societal implications of contemporary ambryo-technologies in farm animals, IETS Satellite Symposium	- Actualización de conocimientos específicos en el campo de aplicación con embriones de animales domésticos	Royal Veterinary and Agricultural University Copenhagen Dinamarca
14 enero-15 febrero 2005	Entrenamiento avanzado en producción de embriones bovines *in vitro*	- Conocer y aplicar conocimientos avanzados en fecundación y cultivo de embriones bovinos *in vitro*	Lyon Experimental Station, University Collage Dublín Dublin, Irlanda

Al comparar las actividades programadas en la propuesta aprobada con las actividades que realmente se realizaron, cuando corresponda, señalar las razones por las cuales algunas de las actividades programadas no se realizaron como estaba previsto o se modificaron.

Incorporar en este punto fotografías relevantes que contribuyan a describir las actividades realizadas.

En términos generales, todas las actividades se realizaron como estaba programado.
En este tipo de trabajo donde el fuerte es en el laboratorio y luego con los animales, que en este caso son donantes o receptores de embriones, ya sea de la misma especie u otra que sirve de "incubadora", regularmente los ensayos de repetición o con diferentes objetivos dentro del mismo tema, se van sobreponiendo. Por ejemplo, los ensayos de fecundación in Vitro se realizaban desde el lunes a miércoles de cada semana, esto ocupa una parte importante del día. El resto de la semana se hace los cultivos y seguimiento de desarrollo o ensayo específicos con los embriones de diferentes estados que se van obteniendo. Durante cada día se programaron seguimientos a animales en tratamientos específicos, por ejemplo de estimulación hormonal en vacas, transferencia de embriones de vaca in Vitro a oviducto de oveja por medio de cirugía etc.

Actividades que se realizaron aprovechando la oportunidad y que no estaban programadas:

- Sincronización de ciclos reproductivos y estimulación de vacas para colección de embriones madurados in vivo
- Seguimiento ecográfico cuantitativo de dinámica ovárica en vacas no estimuladas
- Transferencia quirúrgica de embriones bovino obtenidos in Vitro a oviducto de oveja
- Participación en seminarios semanales de postgrado del departamento de Animal Science, University College Dublín.
- Observación de colección no quirúrgica de embriones bovinos en hembras superovuladas

Contactos Establecidos

Presentar los antecedentes de los contactos establecidos durante el desarrollo de la propuesta (profesionales, investigadores, empresas, etc.), de acuerdo al siguiente cuadro:

Institución/ Empresa/Organi -zación	Persona de Contacto	Cargo	Fono/Fax	Dirección	E-mail
In Vitro Fertilization Laboratory, Deparment of Animal Science, University Collage Dublin	Dr. Patrick Lonergan	Jefe laboratorio	Fono: +353-1-716-7773 Fax: +353-1-716-1103	Lyons Reseach Farm, Newcastle, County Dublin, Ireland	Pat.lonergan@ucd.ie
Deparment of Animal Science and Production and The Conway Institute for Biomedical and Biomolecular Reseach, University College Dublin.	Dra. Trudee Fair	Encargada área de Biología Molecular	Fono: +353-1-601-2147 Fax: +353-1-716-1103	Lyons Reseach Farm, Newcastle, County Dublin, Ireland	trudee.fair@ucd.ie
National Food Centre	Mr. Declan Troy	Director	Fono: +353 18059500, Fax: +353 1 8059550.	Ashtown, Dublin 15 Ireland	dtroy@nfc.teagasc.ie

Material Recopilado

Junto con el informe técnico se debe entregar un set de todo el material recopilado durante la actividad de formación (escrito y audiovisual) ordenado de acuerdo al cuadro que se presenta a continuación (deben señalarse aquí las fotografías incorporadas en el punto 4):

Tipo de Material	Nº Correlativo (si es necesario)	Caracterización (título)
Artículo	-Paper Revistas N° 1 al 6	-In Vitro fertilization and culture of bovina embryos
	- Resumen Protocolos	Media Preparation for bovine IVF embryo production

Foto	Secuencia en CD	- Transferencia de embriones bovinos a oviducto de oveja
Libro, Resumenes Symposium		Agricultural and Societal implications of contemporary embryo.technologies in farm animals
Diapositiva		
CD	1, fotos, charlas, artículos	- CD respaldo Entrenamiento.

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4. PROGRAMA DE DIFUSION EJECUTADO

Programa de difusión ejecutado

En esta sección se deberán describir detalladamente las actividades de difusión realizadas, tales como publicaciones, charlas, seminarios u otras actividades similares, comparando con el programa establecido inicialmente en la propuesta. Se deberá también describir y adjuntar el material de difusión preparado y/o distribuido en dichas actividades.

La información a entregar sobre cada actividad de difusión es la siguiente:

- Tipo de actividad realizada y objetivo principal (incluye elaboración de publicaciones)
- Fecha y lugar de realización
- Temas tratados o exposiciones realizadas
- Destinatarios de la actividad: especificar el tipo y número de personas que asistieron a la actividad (productores, académicos, investigadores, profesionales, técnicos, etc.). Se deberá adjuntar el listado de asistentes según formato indicado más adelante.
- Nombre y tipo de las organizaciones u otras instituciones relevantes en el tema o sector que tuvieron representación en la asistencia al evento.
- Identificación de los expositores que estuvieron a cargo de las presentaciones, indicando su vinculación con la iniciativa y lugar de trabajo
- Indicar si se trató de una actividad abierta a todos los interesados, abierta a quienes se inscribieron previamente, o limitada a quienes fueron especificamente invitados.
- En el caso de los seminarios, deberá adjuntarse el Programa de la actividad que se realizó.

ACTIVIDAD DE DIFUSIÓN

A.- Primera Parte teórica: Viernes 10 de junio de 2005

Charla 1: Principios de fecundación, fecundación in Vitro y biotecnologías en mamíferos

Objetivo: Revisar conocimientos aplicados de Fecundación in Vitro y Biotecnologías asociadas en animales de granja y humano.

Charla 2: Cultivo de embriones in Vitro, principios y actualización

Objetivo: Conocer las condiciones generales de cultivo in Vitro de embriones y los avances en los sistemas con medios semidefinidos, con especial énfasis en el caso del Bovino y comparado al caso humano.

Coordinación: este día se programaron la actividades prácticas a realizar con parte de los asistentes a esta primera parte de la actividad.

Expositores: Las charlas fueron realizadas por el suscrito, con la valiosa participación en las discusiones y preguntas de la Dra. Jennie Rosopatrón G., profesora del programa de Magíster en Biología de la Reproducción de la Facultad de Medicina de la Universidad de la Frontera.

Destinatarios de la Actividad: La primera parte de la actividad fue dirigida a estudiantes de postgrado en el área de Reproducción y estudiantes terminales de la carrera de medicina

veterinaria. No se consideró la participación de productores ya que el fuerte de la actividad es la parte práctica para lo cual no es posible contar con productores. Además, los productores y empresarios del rubro animal ya tuvieron la oportunidad de conocer los aspectos generales de estas tecnologías en las diferentes actividades de difusión que se realizaron en el marco del proyecto FIA en Biotecnología de embriones bovinos recientemente finalizado.

La idea central de la actividad de difusión fue la parte práctica ya que en términos generales la mayor parte de los estudiantes en el área tienen las ideas básicas sin embargo muy pocos han tenido la oportunidad de manejarlo en forma personal. Además creo que es la única forma en que un profesional tenga la posibilidad real de aprender sobre el tema, por eso desde un principio se contempló hacerlo con este enfoque y a un número reducido de personas con cierto grado de formación en el tema.

Para efecto de los estudiantes de postgrado, ellos son profesionales de diferentes carreras, a saber, tecnólogos médicos, profesores, veterinarios y médicos, los que también se desempeñan profesionalmente en distintas instituciones privadas y públicas. Además, esta actividad les fue incluida dentro del programa de formación regular que ellos reciben en la Universidad y también fue evaluada al final de su semestre académico.

A.- Segunda parte, práctica: Miércoles 15 junio al viernes 17 de junio de 2005; lunes 20 y viernes 24 de junio

Programa de actividades y participantes:

Actividad práctica dirigida: La parte práctica se realizó mediante un trabajo dirigido e individual donde participaron 7 personas, 5 estudiantes de postgrado, un profesor de postgrado y un alumno Terminal de pregrado. Cada uno desarrollo un **protocolo y ensayo completo de producción de embriones bovinos in Vitro,** que incluyó las siguientes etapas:

1) Colección y selección de ovocitos obtenidos de ovarios de matadero

2) Maduración de ovocitos

3) Inseminación in Vitro con espermatozoides seleccionados

4) Cultivo in Vitro con medio semidefinido SOF

5) Evaluación de desarrollo embrionario *in vitro*

El procedimiento práctico completo demoró 10 días con un tiempo real de trabajo en laboratorio de 15 a 20 horas

Material entregado en las actividades de difusión

Entregar un listado del material elaborado y distribuido con motivo de la actividad o material audiovisual exhibido como video, datashow, entre otros.

Además, se debe entregar adjunto al informe un set de todo el material entregado en las actividades de difusión (escrito y audiovisual) ordenado de acuerdo al cuadro que se presenta a continuación.

También se deben adjuntar fotografías correspondientes a la actividad desarrollada. El material se debe adjuntar en forma impresa y en un medio magnético (disquet o disco compacto).

Tipo de material	Nombre o identificación	Preparado por	Cantidad
Charlas Datashow	Biotecnologías reproductivas en animales domésticos	Marco Berland O	2 (1 CD)

Participantes en actividades de difusión

Es necesario registrar los antecedentes de todos los asistentes que participaron en las actividades de difusión. El listado de asistentes a cualquier actividad deberá al menos contener la siguiente información:

Nombre	**Fabian Franklin**
Apellido Paterno	**Treulen**
Apellido Materno	**Seguel**
RUT Personal	
Dirección, Comuna y Región	**Senador Estebanez 645, Lab. Jaime Hinostroza** **Temuco, IX región**
Fono y Fax	**45-201056**
E-mail	**Favian_treulen@hotmail.com**
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	**Clínica Alemana de Temuco**

RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	Tecnólogo Médico
Rubro, área o sector a la cual se vincula o en la que trabaja	- Biología Molecular, Citogenética y Citometría de Flujo - Estudiante Magíster Biología de la Reproducción

Nombre	**Mariela Del Carmen**
Apellido Paterno	Muñoz
Apellido Materno	Candia
RUT Personal	
Dirección, Comuna y Región	Cruz 0198, Temuco, IX región
Fono y Fax	45-210882
E-mail	Mariela_CMC@hotmail.com
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	Colegio Providencia "Sagrado Corazón", Temuco
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	Profesor
Rubro, área o sector a la cual se vincula o en la que trabaja	- Biología y Ciencias Naturales - Estudiante Magíster Biología de la Reproducción

Nombre	**Roberto Mauricio**
Apellido Paterno	Valenzuela
Apellido Materno	Viale
RUT Personal	
Dirección, Comuna y Región	Dartnell s/n, Victoria, IX región
Fono y Fax	45-841001
E-mail	robertovalez@hotmail.com
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	Hospital de Victoria
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	- Médico Urólogo
Rubro, área o sector a la cual se vincula o en la que trabaja	- Salud - Estudiante Magíster Biología de la Reproducción

Nombre	**Claudia Esperenza**
Apellido Paterno	Troncoso
Apellido Materno	Muñoz
RUT Personal	
Dirección, Comuna y Región	
Fono y Fax	45-325783

E-mail	troncoso@ufro.cl
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	
Rubro, área o sector a la cual se vincula o en la que trabaja	
Nombre	**Eugenio Felipe**
Apellido Paterno	Garcia
Apellido Materno	Ruíz
RUT Personal	
Dirección, Comuna y Región	Manuel Montt 112, Temuco, IX Región
Fono y Fax	45-325720; 45-325710
E-mail	egarcia@ufro.cl
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	Universidad de la Frontera, Facultad de Medicina
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	Docente tutorial
Rubro, área o sector a la cual se vincula o en la que trabaja	- Docente área morfología y reproducción

	- Estudiante Magíster Biología de la Reproducción
Nombre	**Jennie**
Apellido Paterno	Risopatrón
Apellido Materno	González
RUT Personal	
Dirección, Comuna y Región	Avda. Francisco Salazar 01145, Temuco, IX Región
Fono y Fax	45-325590
E-mail	jennie@ufro.cl
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	Universidad de la Frontera, Facultad de Medicina
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	Académico
Rubro, área o sector a la cual se vincula o en la que trabaja	- Docente Programa Magíster Biología de la Reproducción - Programa de Fecundación Asistida, Clínica Alemana de Temuco
Nombre	
Apellido Paterno	
Apellido Materno	
RUT Personal	

Dirección, Comuna y Región	
Fono y Fax	
E-mail	
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	
Rubro, área o sector a la cual se vincula o en la que trabaja	
OTROS Participantes en parte teórica de difusión: - Paulina Isler García - Michael Oberg Vicent - Mauricio Castillo	 estudiante veterinaria 4° año estudiante veterinaria 4° año estudiante veterinaria, tesista

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Evaluación de las actividades de difusión

Especificar el grado de éxito de las actividades propuestas, señalando las razones de los problemas presentados y sugerencias para mejorarlos en el futuro. Señalar también las razones por las cuales se hicieron modificaciones al programa propuesto inicialmente, en los casos que corresponda.

- La actividad de difusión se desarrolló en el marco de lo planificado y propuesto en la postulación al programa de formación, hubo un número menor de participantes lo cual se debió a diferentes factores a conocer y también su desarrollo se postergó:

a) Se propuso hacer las charlas en las dependencias de la Universidad de la Frontera, lo cual se trasladó a las dependencias de la Escuela de Medicina Veterinaria de la Universidad Católica, para facilitar el trabajo y culminar las presentaciones con una visita a las instalaciones del laboratorio de reproducción, a fin de mostrar el equipamiento y ubicación para las posteriores actividades prácticas.

b) Uno de los profesores de la Universidad de la Frontera asistió a todas las actividades y el resto le fue imposible dejar sus compromisos y otros no se encontraban en Temuco.

c) La actividad se postergó inicialmente para favorecer la asistencia de los estudiantes de postgrado y luego por razones de fuerza mayor.

d) Este tipo de actividades con un fuerte componente práctico requiere de mucho tiempo de dedicación e infraestructura para montarlas por lo cual no puede ser masivo para que sea provechoso

e) Todos los elementos indicados anteriormente están dentro de lo esperado, sin embargo no los acontecimientos que llevaron a postergar la actividad y que han repercutido negativamente en todas las actividades posteriores, particularmente en las propias del ámbito académico del trabajo regular.
La inmanejable necesidad de postergar la actividad de difusión debido a las prolongadas movilizaciones estudiantiles, sumando a la facilidad de puesta en marcha de equipamiento necesario repercutieron fuertemente en el cumplimiento de plazos en el marco de la propuesta. Pese a lo anterior creo que ha sido la mejor forma que esta actividad tenga una repercusión significativa al ser focalizada a personas que realmente están capacitadas e interesadas en conocer y manejar estos conocimientos.

5. EVALUACIÓN DE LA PROPUESTA

Organización durante la actividad (indicar con cruces)[1]

Ítem	Bueno	Regular	Malo
Recepción en país o región de destino según lo programado	x		
Cumplimiento de reserva en hoteles			
Cumplimiento del programa y horarios según lo establecido por la entidad organizadora	x		
Facilidad en el acceso al transporte	x		
Estimación de los costos programados para toda la actividad		x	

En términos generales surgieron algunos inconvenientes en los costos que quiero señalar:

- El costo de vida, alimentación y hospedaje en Europa es muy alto para nuestra realidad
- Los giros de fondos y cambio de moneda tienen un impuesto importante no cosiderado por falta de información al respecto
- Pienso que para personas que viajen a esta zona se debería contar con una estimación más detallada y próxima de los costos de vida, información muy valiosa para no tener problemas. En mi caso particular estos problemas los superé gracias a la excelente voluntad del Dr. Lonergan que me recibió y consiguió un lugar más económico para quedarme.
- En varias oportunidades se hicieron gastos los que no se pudieron respaldar con comprobantes ya que no disponían de ellos o porque simplemente no los entregaban porque no era lo habitual, no tengo claridad cobre las razones de esto en algunos casos.
- Par los efectos de mi estadía en Dublín, el costo total de los gastos de alojamiento fue significativamente menor a lo que sería si se hubiera pagado gastos de hotel o similares. En lo particular se consiguió compartir gastos o subarrendamiento en una casa donde vivían estudiantes de postgrado. Mucos de los gastos eran de tipo fondo común y varios de gastos generales como luz, agua, calefacción etc. En todo caso era un lugar cómodo y mucho más económico que cualquier hotel.

[1] En caso de existir un ítem Malo o Regular, señalar los problemas enfrentados durante el desarrollo de la actividad de formación, la forma como fueron abordados y las sugerencias que puedan aportar a mejorar.

Evaluación de la actividad de formación En esta sección se debe evaluar la actividad en relación a los siguientes aspectos:
a) Efectividad de la convocatoria
Me parece adecuada, sin embargo debo señalar que al momento de postular tuve problemas debido a que se cerró antes el sistema de ventanilla abierta dejando poco margen para completar la documentación necesaria
b) Grado de participación de los asistentes (interés, nivel de consultas, dudas, etc)
- Como una actividad individua, creo que mi participación fue buena y saqué el máximo provecho a mi estadía durante el entrenamiento. En variadas oportunidades acompañé a los estudiantes en sus actividades de madugrada o altas horas de la noche, especialmente en las evaluaciones con animales.
c) Nivel de conocimientos adquiridos en función de lo esperado (se debe indicar si la actividad contaba con algún mecanismo para medir este punto)
Bueno, creo que aprendí más de lo que esparaba y en términos generales la experiencia fue muy enriquecedora para. La actividad no contempló aplicación de algún instrumento de evaluación
d) Calidad de material recibido durante la actividad de formación
El material recibido fue poco ya que el fuerte era la práctica en nuevas metodologías para lo cual el material fue el justo y adecuado, principalmente algunos artículos científicos y un resumen de los protocolos que utilizaban en el laboratorio.

e) Nivel de adecuación y facilidad de acceso a infraestructura/equipamiento necesario para el logro de los objetivos de la actividad de formación.

Conté con todas las facilidades necesarias para tener una estadía provechosa y agradable en mi entrenamiento

f) Indique las materias que fueron más interesantes, más desarrolladas a lo largo de la actividad de formación y las que generan mayor interés desde el punto de vista de la realidad en la cual se desenvuelve el participante.

- Dentro de lo considerado en la formación, uno de los aspectos más interesantes fue el participar y ver las potencialidades asociadas a las técnicas de transferencia interespecífica y evaluación de procedimientos por esta vía. También estas metodologías abren puertas importantes para incursionar en mejoremiento de tecnologías aplicadas al mejoramiento genético animal.

g) Problemas presentados y sugerencias para mejorarlos en el futuro

- No tuve problemas en ninguno de los aspectos propios del programa de formación, excepto con el cumplimiento de la actividad de difusión y derivados de esto.

Aspectos relacionados con la postulación al programa de formación o promoción

a) Apoyo de la Entidad Patrocinante (cuando corresponda)

___X_ bueno ____ regular ____ malo

Justificar: se me entregaron los recursos solicitados y se realizó una rápida tramitación de los documentos necesarios para la postulación, pese a la premura del tiempo.

b) Información recibida por parte de FIA para realizar la postulación

____ amplia y detallada ___x_ aceptable ____ deficiente

Justificar: Fue un problema el hecho de haber acortado los plazos de postulación ya que casi no me dejo margen para completar los trámites necesarios

c) Sistema de postulación al Programa de Formación o Promoción (según corresponda)

___x_ adecuado ____ aceptable ____ deficiente

Justificar: Me parece que el sistema de ventanilla abierta es adecuado y los documentos exigidos también lo son

d) Apoyo de FIA en la realización de los trámites de viaje (pasajes, seguros, otros) (sólo cuando corresponda)

___x_ bueno ____ regular ____ malo

Justificar: Todos los trámites de pasajes y seguros fueron realizados de buena forma y siempre considerando mis sugerencias para mayor comodidad

e) Recomendaciones (señalar aquellas recomendaciones que puedan aportar a mejorar los aspectos administrativos antes indicados)

- En mi programa, además del entrenamiento se me aceptó mi participación en el congreso anual de la IETS. Creo que este evento debería estar dentro de la lista de apoyo a eventos permanentes del FIA ya que me parece que es el único relacionado a aspectos de vanguardia en el tema animal. Este congreso se realiza anualmente en algún país del mundo desarrollado.
- También creo que sería útil que le FIA hiciera alguna sugerencia a los postulantes en el sentido de combinar actividades, en la medida de lo posible, de modo de sacar más provecho a los recursos invertidos.

ANEXO
MATERIAL RECOPILADO

Artigo nº 1.

ELSEVIER

Theriogenology

Theriogenology 57 (2002) 2105–2117

Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire

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Abstract

The aim of these experiments was to investigate the effect of duration of IVM, duration of gamete co-incubation, and of sperm dose on the development of bovine embryos in vitro. In addition, the speed of sperm penetration of six bulls of known differing in vivo and in vitro fertility was examined. In Experiment 1, following IVM for 16, 20, 24, 28 or 32 h, cumulus oocyte complexes (COCs) were inseminated with 1×10^6 spermatozoa/ml. After 24 h co-incubation, presumptive zygotes were denuded and placed in droplets of synthetic oviduct fluid (SOF). In Experiment 2, following IVM and IVF, presumptive zygotes were removed from fertilization wells at 1, 5, 10, 15 or 20 h post insemination and placed in culture as described above. In Experiment 3, following IVM, COCs were inseminated with sperm doses ranging from 0.01×10^6 to 1×10^6 spermatozoa/ml. Following co-incubation for 24 h, presumptive zygotes were placed in culture as described above. In Experiment 4, following IVM, oocytes were inseminated with sperm from six bulls of known differing field fertility. To assess the rate of sperm penetration, oocytes were subsequently fixed every 3 h (up to 18 h) following IVF. Based on the results of Experiment 4, in Experiment 5, following IVM for 12, 18 or 24 h, COCs were inseminated with sperm from two sires with markedly different penetration speeds. After 24 h co-incubation, presumptive zygotes were denuded and placed in culture. The main findings from this study are that (1) the optimal duration of maturation of bovine oocytes in vitro to maximize blastocyst yield is 24 h, (2) sperm-oocyte co-incubation for 10 h is sufficient to ensure maximal blastocyst yields, (3) sperm concentrations of 0.25×10^6 and 0.5×10^6 spermatozoa/ml yielded significantly more blastocysts than any other concentration within the range of 0.01×10^6–1×10^6 spermatozoa/ml, (4) there are marked differences in the kinetics of sperm penetration between sires and this may be a useful predictor of field fertility, and (5) the inferior development associated with slower penetration rates may in part be overcome by carrying out IVF at a time when

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of bovine oocytes. In addition, the relationship between the rate and kinetics of sperm penetration and bull fertility in vitro and in vivo was assessed using semen from bulls of known field fertility.

2. Materials and methods

2.1. Oocyte collection and in vitro maturation (IVM)

Chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Cumulus oocyte complexes (COCs) were obtained by aspiration from 2 to 8 mm follicles from the ovaries of slaughtered cows within a maximum of 3 h after slaughter. After four washes in modified PBS supplemented with 36 μg pyruvate/ml, 50 μg gentamycin/ml and 0.5 mg bovine serum albumin (BSA)/ml (Sigma fraction V, A-9647), groups of approximately 50 COCs were placed in 500 μl maturation medium in four-well dishes (Nunc, Roskilde, Denmark) and cultured for 24 h (unless otherwise stated) at 39 °C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng epidermal growth factor/ml.

2.2. Semen source, sperm preparation and in vitro fertilization (IVF)

Following maturation, COCs were washed four times in PBS and then in fertilization medium. COCs were placed in four-well dishes containing 250 μl of fertilization medium (TALP containing 10 μg heparin-sodium salt/ml, 184 IU heparin/mg, Calbiochem, San Diego, CA, USA) per well. Motile spermatozoa were obtained by centrifugation of frozen-thawed semen on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (2.5 ml 45% (v/v) Percoll over 2.5 ml 90% (v/v) Percoll) for 8 min at 700 × *g* at room temperature. Viable spermatozoa, collected at the bottom of the 90% fraction were washed in Hepes-buffered TALP and pelleted by centrifugation at 100 × *g* for 5 min. Spermatozoa were counted in a hemocytometer and diluted in the appropriate volume of fertilization medium to give a concentration of 2×10^6 spermatozoa/ml (unless otherwise stated). A 250 μl aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/ml. Unless otherwise stated, plates were incubated for approximately 24 h at 39 °C under an atmosphere of 5% CO_2 in air with maximum humidity. In the experiments described below, the time of addition of the spermatozoa to the oocytes is defined as the time of insemination (0 h).

2.3. In vitro culture (IVC)

Embryo culture was carried out in modified synthetic oviduct fluid (SOF) under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39 °C [21]. At 24 h after insemination, presumptive zygotes were denuded of cumulus cells by vortexing for 2 min in 2 ml of PBS. The zygotes were washed four times in PBS and once in SOF before they were transferred to 25 μl culture droplets. 24 h after placement in culture (i.e. 48 h after insemination) 10% (v/v) FCS was added to the droplets.

known [18]. Following IVM for 24 h, oocytes were randomly assigned to one of six bulls for IVF (nonreturn rates ranging from 78 to 56%) and were inseminated with MitoTracker-stained sperm from each bull as described above. Presumptive zygotes were removed from the fertilization drops at 3, 6, 9, 12, 15 or 18 hpi. Cumulus cells were removed by pipetting, and the zona pellucida was digested using 0.5% protease. Zona-free oocytes were placed on a glass slide, allowed to air dry and were fixed in ethanol overnight. They were subsequently stained with Hoechst 3334L or Propidium iodide and examined using a confocal laser scanning microscope system (BioRad, Hemelhempstead, UK) for evidence of sperm penetration (Fig. 1). To demonstrate that MitoTracker-staining did not affect

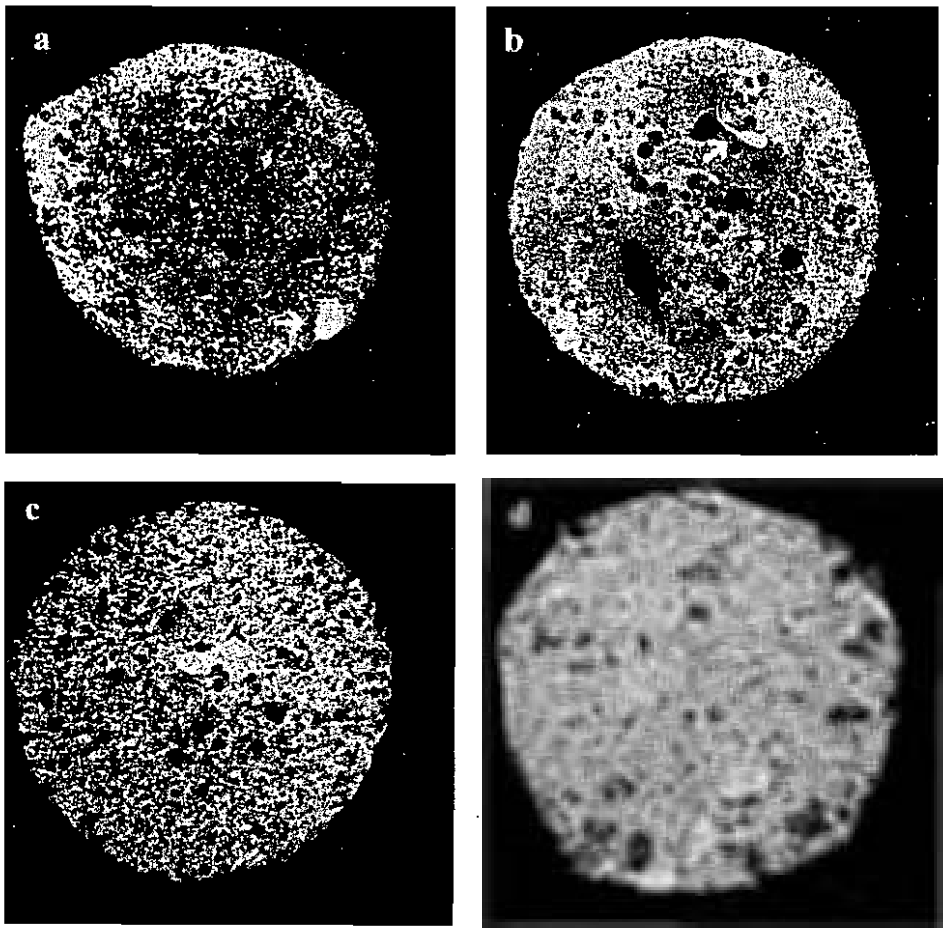

Fig. 1. Representative images showing various stages of sperm penetration. The zona was removed prior to fixation. (a) Non fertilized oocyte 3 h after insemination showing metaphase plate; (b) fertilized oocyte 3 h after insemination. Note sperm (arrow) and chromosomes arranged at anaphase/telophase II prior to expulsion of second polar body; (c) fertilized oocyte 9 h after insemination. Note two pronuclei and sperm tail; (d) polyspermic oocyte 6 h after insemination. Note sperm tails (arrows) lying adjacent to decondensing sperm heads.

Table 2
Effect of gamete co-incubation time on subsequent development of bovine oocytes in vitro (Experiment 2)

Sperm-oocyte co-incubation (h)	Total no. of oocytes	Oocytes cleaved		Day 8 blastocyst yield	
		n	%	*n*	%
1	255	4	1.6^{d}	0	0.0^{c}
5	240	89	37.1^{c}	46	19.2^{b}
10	254	164	64.6^{b}	82	32.3^{a}
15	255	209	82.0^{ab}	103	40.4^{a}
20	251	215	85.7^{a}	80	31.9^{a}

Values in the same column with different superscripts (a, b, c, d) differ significantly ($P < 0.05$).

3.2. Experiment 2

Following co-incubation for 1 h, almost all oocytes (98.5%) were unfertilized (Table 2). While a co-incubation time of 5 h resulted in a significant increase in cleavage rate, the percentage of oocytes undergoing cleavage was still significantly lower than in all other treatments. Increasing the co-incubation time to 10 h significantly increased the cleavage rate, but the value was still significantly lower than that achieved with 20 h co-incubation. No blastocysts developed following co-incubation for 1 h, and although co-incubation for 5 h resulted in a 16% blastocyst yield, this was significantly lower than all other treatments of a longer duration. There was no significant difference in blastocyst development following gamete co-incubation for 10, 15 or 20 h.

3.3. Experiment 3

There were no significant differences in cleavage rate following insemination with 1×10^6 compared to 0.50×10^6, 0.25×10^6, 0.125×10^6 or 0.05×10^6 spermatozoa/ml (Table 3). However, insemination with 0.50×10^6 spermatozoa/ml significantly increased cleavage when compared to 0.125×10^6, 0.05×10^6 or 0.01×10^6 spermatozoa/ml. ($P < 0.05$). When the sperm dose was lowered to 0.01×10^6 spermatozoa/ml cleavage rate was significantly reduced compared to all other treatments ($P < 0.0001$). Blastocyst

Table 3
Effect of sperm dose in vitro on fertilization and subsequent development of bovine oocytes to the blastocyst stage (Experiment 3)

Sperm concentration (million/ml)	Sperm:oocyte	Total no. of oocytes	Oocytes cleaved		Day 8 blastocyst yield	
			n	%	*n*	%
1.0	10000:1	237	185	78.1^{ab}	59	24.9^{cd}
0.5	5000:1	212	179	84.4^{a}	75	35.4^{bc}
0.25	2500:1	225	179	79.6^{ab}	66	29.3^{cd}
0.125	1250:1	238	179	75.2^{b}	58	24.4^{de}
0.05	500:1	213	155	72.8^{b}	45	21.1^{ee}
0.01	100:1	203	100	49.3^{c}	12	5.9^{f}

Values in the same column with different superscripts (a, b, c, d, e, f) differ significantly ($P < 0.05$).

Table 5
Correlation between time of sperm penetration in vitro and field fertility for six bulls (150-day nonreturn rate; $n = 18$ batches: six bulls, three replicates/bull)

Time of sperm penetration (hpi)	Correlation coefficient and significance	
	r	P-value
3	0.827	0.043
6	0.671	0.145
9	0.712	0.112
12	0.676	0.141

hpi: hours post insemination.

Table 6
Effect of time of insemination on blastocyst yield using two sires (Experiment 5)

Sire	Time of insemination[1]	Total no. of oocytes	% cleaved (48 hpi)	Day 8 blastocyst yield
HTA	12	238	63.9[a]	18.1[a]
	18	240	80.4[b]	24.2[ab]
	24	246	81.7[b]	29.7[b]
TJD	12	247	35.6[a]	10.9[a]
	18	247	64.8[b]	16.6[a]
	24	242	62.8[b]	11.2[a]

Values in the same column for the same sire with different superscripts (a, b) differ significantly ($P < 0.05$).
[1] Relative to start of IVM.

between inseminations at 12 and 24 h. For TJD, there was no significant difference in blastocyst yield between any of the treatments (Table 6).

4. Discussion

The main findings from this study are that (1) the optimal duration of maturation of bovine oocytes in vitro to maximize blastocyst yield is 24 h, (2) sperm-oocyte co-incubation for 10 h is sufficient to ensure maximal blastocyst yields, (3) a sperm concentration below 0.25×10^6 spermatozoa/ml resulted in significantly less blastocysts than any concentration higher than this, (4) there are marked differences in the speed of sperm penetration between sires of differing in vitro and in vivo fertility, and (5) the inferior development associated with slower penetration rates may in part be overcome by carrying out IVF at a time when the actual penetration is most likely to coincide with the completion of maturation.

Maturation of the oocyte is not required for sperm penetration or for sperm nuclear decondensation under in vitro conditions [6,7]. However, premature exposure to sperm in general leads to reduced development. The kinetics of bovine IVM have been reported by several authors [3–5]. In general, under the conditions of IVM used in the present study, by 16 h approximately 50% of oocytes are at telophase I (in the process of polar body

In vivo, the ovulated bovine oocyte encounters few free-swimming sperm at the site of fertilization. In fact, fertilization is likely to occur in conditions where the sperm:oocyte ratios are close to 1:1 [20]. In bovine IVF, commonly used sperm concentrations range from 0.50×10^6 to 5×10^6 spermatozoa/ml [1]. The sperm concentration can significantly influence the proportion of oocytes undergoing cleavage and development to the blastocyst stage. A compromise almost certainly must be found between having a sufficient number of sperm to ensure acceptable fertilization rates while ensuring a minimal incidence of polyspermy.

In the present study, a concentration of 0.125×10^6–0.5×10^6 spermatozoa/ml provided enough spermatozoa to optimize blastocyst development and was superior in terms of cleavage rate to all concentrations below 0.25×10^6 spermatozoa/ml. These data are in agreement with those of Long et al. [24] who indicated that even at reduced sperm concentrations, there was no increase in the proportion of oocytes inseminated by only one spermatozoon. However, a concentration of 0.50×10^6 is superior to a concentration of 1.0×10^6 spermatozoa/ml. While some authors have reported an increase in the rate of polyspermy at high concentrations and high sperm:oocyte ratios [6,12] the incidence of polyspermy in this experiment was low (1–8%) and not significantly different across all treatments (data not shown).

It is well established that the bull can have a major effect on the outcome of IVF [17,18,32,33]. We have previously shown that the time of first oocyte cleavage varies between different bulls and that the kinetics of early cleavage can be used to discriminate between bulls of high and low field fertility [18]. Of the bulls tested in that study, HTA was ranked the highest, and TJD the lowest where the time of first cleavage was used as the basis for predicting field fertility. The strongest correlation between cleavage and field fertility was observed at 33 hpi. For HTA >60% of oocytes inseminated had cleaved by this time and these accounted for almost 70% of all blastocysts from this bull, while in contrast for TJD, the lowest ranked bull, only 15% of oocytes had cleaved by 33 hpi. These marked differences in early cleavage kinetics were the basis of Experiment 4, where we investigated whether speed of oocyte penetration is correlated with field fertility. The results indicate that there is a marked difference in the speed of oocyte penetration between different bulls which may partially explain the observed differences in cleavage rate, blastocyst yield and field fertility. Bulls with a high nonreturn rate (ELG, HTA and LEW) displayed the fastest rate of sperm penetration while those ranked lowest in field fertility (MTL, KKN and TJD) had slower rates of penetration. The differences in speed of sperm penetration were most obvious after 3 h gamete co-incubation, and this is reflected in the correlation coefficient between nonreturn rate and percentage oocytes penetrated by 3 hpi ($r = 0.827$, $P < 0.05$). This data is consistent with our previous study [18]. This would imply that sperm which penetrate faster lead to early cleaving embryos, which have been shown to be developmentally superior to those embryos which cleave later [34].

The timing of insemination (i.e. the addition of the sperm to the oocyte) in vitro is clearly not as important as the actual time of sperm penetration of the oocyte. On the basis of these results, Experiment 5 was carried out to try and overcome the slow penetration rate of TJD by inseminating earlier during IVM. The results demonstrate that for a bull with a "normal" speed of penetration (HTA), insemination earlier during IVM does not have any beneficial effect; in fact, insemination at 12 h resulted in significantly poorer development. In contrast,

[15] Quinn P, Lydic ML, Ho M, Bastuba M, Hendee F, Brody SA. Confirmation of the beneficial effects of brief co-incubation of gametes in human in vitro fertilization. Fertil Steril 1998;69:399–402.
[16] Lin SP, Lee RK, Su JT, Lin MH, Hwu YM. The effects of brief gamete co-incubation in human in vitro fertilization. J Assist Reprod Genet 2000;17:344–8.
[17] Marquant-Le Guienne B, Humblot P, Thibier M, Thibault C. Evaluation of bull semen fertility by homologous in vitro fertilization tests. Reprod Nutr Dev 1990;30:259–66.
[18] Ward FA, Rizos D, Corridan D, Quinn K, Boland MP, Lonergan P. Paternal influence on the time of the first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo. Mol Reprod Dev 2001;60:47–55.
[19] Otoi T, Tachikawa S, Kondo S, Suzuki T. Effects of different lots of semen from the same bull on in vitro development of bovine oocytes fertilized in vitro. Theriogenology 1993;39:713–8.
[20] Hunter RHF. Sperm:egg ratios and putative molecular signals to modulate gamete interactions in polytocous mammals. Mol Reprod Dev 1993;35:324–7.
[21] Carolan C, Lonergan P, Van Langendonckt A, Mermillod P. Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in vitro. Theriogenology 1995; 43:1115–28.
[22] Monaghan P, Carolan C, Lonergan P, Sharif H, Wahid H, Gordon I. The effect of maturation time on the subsequent in vitro development of bovine oocytes. Theriogenology 1993;39:270. [abstract].
[23] Semple E, Loskutoff N, Leibo SP, Betteridge KJ. Effects of culture medium and maturation time on in-vitro development of bovine oocytes into blastocysts. Theriogenology 1993;39:307. [abstract].
[24] Long CR, Damiani P, Pinto-Correia C, MacLean RA, Duby RT, Robl JM. Morphology and subsequent development in culture of bovine oocytes matured in vitro under various conditions of fertilization. J Reprod Fertil 1994;102:361–9.
[25] Hunter RHF. Aging of the unfertilised cow egg in vivo: how soon is fertility compromised. Vet Rec 1989;124:489–90.
[26] Hunter RHF, Greve T. Could artificial insemination of cattle be more fruitful? Penalties associated with aging eggs. Reprod Dom Anim 1997;32:137–41.
[27] Gliedt DW, Rosenkrans Jr. CF, Rorie RW, Rakes JM. Effects of oocyte maturation length, sperm capacitation time, and heparin on bovine embryo development. J Dairy Sci 1996;79:532–5.
[28] Hunter RHF, Wilmut I. Sperm transport in the cow: periovulatory redistribution of viable cells within the oviduct. Reprod Nutr Dev 1984;24:597–608.
[29] Wilmut I, Hunter RHF. Sperm transport in the oviducts of heifers mated early in oestrus. Reprod Nutr Dev 1984;24:461–8.
[30] Kochhar HPS, Buckrell BC, Pollard JW, King WA. Cleavage, sex and cell number of ovine embryos developed after different sperm-oocyte co-incubation intervals. Theriogenology 1999;51:322. [abstract].
[31] Lin SP, Lee RK, Su JT, Lin MH, Hwu YM. The effects of brief gamete co-incubation in human in vitro fertilization. J Assist Reprod Genet 2000;17:344–8.
[32] Hillery FL, Parrish JJ, First NL. Bull specific effect on fertilization and embryonic development in vitro. Theriogenology 1990;33:249. [abstract].
[33] Shi DS, Lu KH, Gordon I. Effects of bulls on fertilization of bovine oocytes and their subsequent development in vitro. Theriogenology 1990;33:324. [abstract].
[34] Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex and pregnancy rates following transfer of bovine preimplantation embryos. J Reprod Fertil 1999;117:159–67.

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M. BERLAND

ELSEVIER

HIGH BOVINE BLASTOCYST DEVELOPMENT IN A STATIC IN VITRO PRODUCTION SYSTEM USING SOFaa MEDIUM SUPPLEMENTED WITH SODIUM CITRATE AND MYO-INOSITOL WITH OR WITHOUT SERUM-PROTEINS

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ABSTRACT

We describe a bovine embryo culture system that supports repeatable high development in the presence of serum or BSA as well as under defined conditions in the absence of those components. In the first experiment, embryo development in SOF with amino acids (SOFaa), sodium citrate (SOFaac) and myo-inositol (SOFaacI) and with BSA or polyvinyl alcohol (PVA) was compared with that in a M199 granulosa cell co-culture (M199 co-culture). Subsequently, development and cell numbers of blastocysts cultured under defined conditions in SOFaacI with PVA (SOFaacI-PVA), or under undefined conditions in SOFaacI with 5% cow serum (SOFaacI-CS) or M199 co-culture were compared. The repeatability of culture results in SOFaacI-CS was checked by weekly replicates (n=30) spread over 11 months. The viability of embryos developed in SOFaacI-PVA was estimated by transfer of morphologically good blastocysts (n=10) to synchronized recipients. In the second experiment, the effect of omitting CS or BSA from IVM and IVM-IVF on subsequent embryo development in SOFaacI-PVA or in SOFaacI-CS was investigated. Blastocyst development in SOFaa-PVA, SOFaac-PVA, SOFaa-BSA and M199 was 16 ± 3^{b}, 23 ± 2^{ab}, 30 ± 8^{a} and 36 ± 7^{a}%, respectively ($P^{ab} < 0.05$). Additional inclusion of myo-inositol resulted in 42 ± 1^{a}% blastocysts in SOFaacI-PVA vs 19 ± 3^{b}% in SOFaac-PVA, 47 ± 7^{a}% in SOFaac-BSA, and 39 ± 7^{a}% in M199 co-culture, respectively ($P^{ab} < 0.01$). In 30 replicates, the average cleavage and blastocyst rates of oocytes in SOFaacI-CS were 87 ± 4 and 49 ± 5%, respectively. Five normal calves were produced after transfer of 10 blastocysts developed in defined culture medium (i.e., SOFaacI-PVA). Defined IVM or IVM-IVF (i.e., in absence of CS and BSA) reduced cleavage rates (83 ± 3 and 56 ± 3% vs 90 ± 1% in presence of CS; $P < 0.01$). Subsequent embryo development in SOFaacI-CS was not affected in either of these defined conditions. However, cleavage and blastocyst rates under completely defined IVP conditions were 54 ± 7 and 19 ± 4%, respectively. It was concluded that under defined culture conditions, addition of citrate and myo-inositol improved blastocyst development to rates comparable to those obtained with serum, BSA or co-culture and that the quality of blastocysts was not affected by the absence of serum or BSA. However, serum was essential during IVM/IVF for normal fertilization and subsequent high blastocyst development.

Key words: cattle, IVP, embryos, synthetic oviduct fluid, defined medium

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INTRODUCTION

In vitro production of bovine embryos for breeding purposes requires that the culture system and its components are free of specific diseases and support high embryo development with repeatable results. Defined culture conditions supporting high embryo development rates without inclusion of biological substances like serum and bovine serum albumin (BSA) are therefore advantageous and would allow for better quality control and ease of comparison of the results between laboratories and experiments. In addition, a simplified in vitro production system could be used outside laboratory conditions.

A variety of culture conditions allow for embryo development to the blastocyst stage (for review, see 3). In a weighted analysis of blastocyst development published in abstracts (at the IETS-meetings 1991 to1995; 50), the most significant variance was attributed to the random effect of between abstract variation, although the inclusion of somatic cells also had a positive effect on the overall development rate of blastocysts.

Co-culture with somatic cells (e.g., oviduct epithelial cells, granulosa cells or BRL-cells) is essential for achieving acceptable rates of embryo development under certain conditions, (1, 16, 7, 9). However, addition of cells to a culture medium renders it an undefined medium, which carries a certain risk of infecting the embryos with pathogens (2, 44). The risk of infection is higher for IVP embryos as certain pathogens cannot be removed by standard IETS-washing procedures (51). Both BSA and serum, which are components of most embryo culture media, are complex and undefined mixtures of proteins, growth factors, peptides and the like. Somatic cells also lack adequate definition to assure optimal quality control. Furthermore, serum is suspected of contributing to the large offspring syndrome in sheep (26, 49, 43). Serum and BSA also have a stimulatory effect on embryo growth (37, 26, 25, 17). Factors leading to improved embryo development in culture systems using somatic cells and/or serum-proteins are not well understood, but include positive embryotrophic factors and inactivation of embryotoxic agents (e.g., free radicals, heavy metals and others, see 3). In BSA, citrate has been identified as a positive embryotrophic factor (20), but BSA may also stimulate embryo development through amelioration of embryotoxic substances (15). With the addition of amino acids, BSA can be used to substitute for serum in embryo culture media without compromising blastocyst rates (17).

Bovine oocytes have been shown to mature (3, 13, 38) and fertilize (29, 39, 45) in vitro in the absence of serum-proteins without compromising subsequent embryo development in vitro. Bovine zygotes have also been reported to develop, with various rates of success, in chemically defined medium lacking serum proteins (modified SOF: 22, 29, 30, 32; M199: 11, 36). However, chemically defined conditions allow for more precise observation of the effect of supplementation on a given medium. Embryo development has been significantly improved by conditioning with somatic cells (34) or by the inclusion of growth or other embryotrophic factors such as citrate (30, 22), myo-inositol (P. Kotaris, personal communication; 22), EGF and FDF (32) or PDGF (12).

The viability of embryos produced under conventional in vitro culture systems is generally lower than that of in vivo produced embryos (40 to 50% vs. 50 to 75% survival following transfer; for review see 22). Data regarding the transfer of embryos produced in defined medium are limited, and only a few pregnancies have been reported (30).

In the present study, bovine blastocyst development was investigated following: 1) the inclusion of citrate and myo-inositol during in vitro culture in a modified, chemically-defined

synthetic oviduct fluid medium (SOFaaci; Table 1), and 2) the omission of serum and BSA from in vitro maturation and fertilization media and culture in M199 granulosa co-culture (M199 co-culture). In SOF-medium with 5% cow serum (SOFaaci-CS) or in chemically defined SOF-medium (SOFaaci-PVA). We also report on pregnancies and birth of live calves following transfer of IVP-blastocysts cultured in vitro from the 1-cell stage in SOFaaci-PVA. Part of these data has been published earlier in abstract form (22).

Table 1. Composition of modified synthetic oviduct fluid (SOFaaci) used in present study[abcd]

Component	Product, Company	Concentration
NaCl	S 5886, Sigma	107.63 mM
KCl	P 5405, Sigma	7.16 mM
KH_2PO_4	P 5655, Sigma	1.19 mM
$MgSO_4$	M 2643, Sigma	1.51 mM
$CaCl_2.2H_2O$	C 7902, Sigma	1.78 mM
Sodium lactate	L 4263, Sigma	5.35 mM
$NaHCO_3$	S 4019, Sigma	25.00 mM
Na - pyruvate	P 3662, Sigma	7.27 mM
l-Glutamine[b]	G 6392, Sigma	0.20 mM
BME amino acids[b]	B 6766, Sigma	45.0 µL/mL
MEM amino acids[b]	M 7145, Sigma	5.0 µL/mL
tri-Sodium-citrate[c]	1.06448, Merck	0.34 mM
Myo-inositol[d]	I 7508, Sigma	2.77 mM
Gentamycine	G 1264, Sigma	50.0 µg/mL
Phenol-red	P 5530, Sigma	10.0 µg/mL
H_2O	W 1503, Sigma	

[a]The osmolarity of SOFaaci with or without supplements (see text) were adjusted to 270 to 280 mOsm. Medium was stored at 5°C for a maximum of one week.
[b]SOF including amino acids (but excluding citrate and myo-inositol) is designated as SOFaa.
[c]SOFaa including citrate is designated as SOFaac.
[d]SOFaac including myo-inositol is designated as SOFaaci.

MATERIALS AND METHODS

The study was carried out between 1995 and 1998. The initial standard procedures for in vitro maturation (IVM), fertilization (IVF) and culture (IVC) have been described earlier (53). However, in 1997, the standard co-culture procedure using M199 with granulosa cells was replaced by SOF-medium (47) supplemented with amino acids, citrate and myo-inositol (Table 1) and 5% cow serum (SOFaaci-CS). The cow serum (CS; Danish Veterinary Laboratory, Frederiksberg, Denmark) used in our present study originated from 2 batches, tested and preselected for their ability to support blastocyst development in vitro. Likewise, the batch of highly purified low endotoxin, gamma-irradiated and IgG-free bovine serum albumin (BSA; cat. no. ABRZ, Immuno-Chemical Products Ltd., Auckland, New Zealand) had also been tested and preselected (25). Media that do not contain CS or BSA are

considered defined media. Both IVF and IVC media have been thoroughly chemically characterized. However, defined IVM medium contains eCG and hCG, which despite the high degree of purity are biological substances. An overview of the different experiments is presented in Table 2.

Table 2. Overview of experiments and media

Experiment		Media		
No	Description	IVM[a]	IVF[b]	IVC[c]
1	Citrate in defined IVC	M199-CS	TLP-CS	SOFaa-BSA, SOFaa-PVA ± citrate or M199 co-culture
2a	Myo-inositol in defined vs in undefined IVC	M199-CS	TLP-CS	SOFaac-BSA ± myo-inositol, SOFaac-PVA ± myo-inositol or M199 co-culture
2b	In vivo survival of blastocysts developed in defined IVC	M199-CS	TLP-CS	SOFaaci-PVA
3a	Defined IVC vs undefined IVC	M199-CS	TLP-CS	SOFaaci-PVA, SOFaaci-CS or M199 co-culture
3b	Stability of the SOFaaci-CS system	M199-CS	TLP-CS	SOFaaci-CS
4a	High vs low serum concentrations vs BSA in IVF	M199-CS	TLP-CS (1%), TLP-10%CS or TLP-BSA	M199 co-culture -
4b	Defined IVM vs defined IVM-IVF vs undefined IVM-IVF	M199-CS or M199	TLP-CS or TLP	SOFaaci-CS
5	Defined IVC vs defined IVP vs undefined IVP	M199-CS or M199	TLP-CS or TLP	SOFaaci-PVA or SOFaaci-CS

[a]IVM-medium contained eCG and hCG. In defined conditions 0.2 to 0.3 mg/mL PVA was present, carried over from the aspiration / washing medium.
[b]IVF-medium contained heparin, hypotaurine, epinephrine and penicillamine.
[c]See Table 1 for detailed composition of SOF-media. See reference 53 for details of M199 co-culture.

Oocyte Recovery and In Vitro Maturation

Ovaries were collected from cows slaughtered at a nearby slaughterhouse and transported to the laboratory within 2 to 4 h in 0.9% NaCl-solution (Pharmacia AS, Copenhagen, Denmark) at 28 to 30° C. Cumulus-oocyte-complexes (COC) were aspirated from follicles 2 to 6 mm in diameter with a 19-g needle into Hepes-buffered M199 (M0650, Sigma, St. Louis, MO, USA) supplemented with 5 IU/mL heparin (LEO Chemical Factory, Ballerup, Denmark), 2.5 mg/mL amphotericin (A2942, Sigma) and 1% CS. The COC's with

intact tight cumulus cell layers were selected for IVM, then washed once in aspiration medium and transferred in groups of 25 to 30 per well of 4-well dishes (176740, Nunc™, Life Technologies AS, Roskilde, Denmark) containing 450 µL IVM medium (bicarbonate-buffered M199 [S0148; Sigma]) supplemented with 10 IU/mL eCG and 5 IU/mL hCG (constituents of Suigonan®; Intervet Scandinavia, Skovlunde, Denmark), 0.4 mM l-glutamine (G5763; Sigma) and 50 µg/mL gentamycin (G1264; Sigma). The standard IVM-medium (M199-CS) was further supplemented with 15% CS. Immature COC's were incubated for 22 to 25 h under paraffin oil (Uvasol, 1.07161.0500; Merck KGaA, Darmstadt, Germany) at 38.6° C in 5% CO_2 in humidified air.

In the IVM trials where COC's were matured under defined conditions in M199 (Table 2; Experiments 4b, 5), serum was not present in the medium and was not substituted for by any other macromolecular substances. However, because CS was substituted with 3 mg/mL polyvinyl alcohol (PVA; P8236; Sigma) in the aspiration and washing medium to minimize stickiness of the COC's, minimal amounts of PVA (0.2 to 0.3 mg/mL final concentration) were carried over into the IVM-culture when the COC's were transferred to maturation dishes.

Sperm Preparation and In Vitro Fertilization

Frozen-thawed semen from two Danish Holstein-Friesian bulls of proven fertility was used for IVF (one in 1995 to 96, the other in 1997 to 98). The thawed semen was loaded on the top of a discontinuous Percoll gradient in a 10-mL conical tube (347694, Nunc™) and centrifuged for 25 min at 200 g at ambient temperature (≈ 23° C). The gradient consisted of 2 mL 55% and 2 mL 90% Iso-Percoll (Pharmacia AB, Stockholm, Sweden) dissolved in modified Tyrode's solution (35) without glucose or BSA (Sperm-TLP). The pellet was resuspended and centrifuged twice in 2 mL Sperm-TLP at 200 g for 10 min. After the final wash, the pellet was resuspended in 100 µL Sperm-TLP, and IVF was performed in 300 µL IVF-medium (TLP; modified TALP-medium for fertilization [35] without BSA or glucose) supplemented with 5 IU/mL heparin (H3149, Sigma), 10 µM penicillamine (P4875, Sigma), 15 µM hypotaurine (H1384, Sigma) and 1 µM epinephrine (E4250, Sigma). In the standard IVF-protocol, mature COC's were transferred to IVF-wells without washing. Consequently, the standard IVF medium (TLP-CS) contained ≈1% CS originating from the standard IVM-medium (M199-CS). Twenty microliters of the sperm suspension was finally added to the IVF-wells, producing a final sperm concentration of ≈1.5 x 10^6 cells/mL. The gametes were co-cultured under paraffin oil for 20 h at 38.6° C in 5% CO_2 in humidified air.

In IVF experiments with defined medium (Table 2; Experiments 4b, 5), the COC's were washed 3 times in Hepes-buffered M199 without serum, BSA or any other macromolecular supplement prior to transfer to the IVF wells.

In Vitro Culture

Presumptive zygotes were vortexed at 3000 rpm for 90 sec in 0.5 mL Hepes-buffered M199 with 10% CS in a 6-mL tube (2003; Falcon®; Becton Dickinson AB, Stockholm, Sweden). The zygotes were recovered and transferred without further washing to 4-well dishes in groups of 30 to 50 per well for IVC under paraffin oil.

For M199 co-culture, the original maturation dishes were used after replacing the IVM-medium with 400 µL bicarbonate buffered M199 with 5% CS at the time of insemination and allowing the remaining granulosa cells to form a monolayer. At 96 h post insemination, ova

were transferred to the other wells of the dish in which CS was added for a final concentration of 10% serum (53).

For culture in SOF-media, dishes contained 400 µL medium and were incubated for 7 to 9 d post insemination in a humidified mixture of 5% O_2, 5% CO_2 and 90% N_2 air. The standard SOF-medium contained 5% CS (i.e., SOFaaci-CS). In IVC experiments using defined SOF-media (i.e., SOFaa-PVA, SOFaac-PVA, SOFaaci-PVA; Table 2, Experiments 1, 2, 3, 5), 3 mg/mL PVA were added as a macromolecule, and zygotes were vortexed and washed 3 times in defined Hepes-buffered M199 with PVA prior to IVC.

Cleavage was recorded once between Day 2 and 4, and blastocyst numbers at Day 7 and/or Day 9 post insemination. Hatching of blastocysts was recorded at Day 9 after insemination.

Staining of Ova

Random samples of both mature and fertilized oocytes were freed from cumulus cells by vortexing, mounted on glass slides and fixed in 3:1 ethanol:acetic acid after 24 h of IVM or at 20 h post insemination, respectively. The fixed oocytes were stained in 1% aceto-orcein and analyzed at x 400 magnification to determine the nuclear maturation (metaphase II after extrusion of the first polar body) or normal fertilization (2 pronuclei and presence of sperm tail).

To determine cell numbers, blastocysts were transferred to fixation medium (3:1 ethanol:acetic acid). Groups of 5 to 10 embryos were transferred to a glass slide in 2 to 5 µL of fixation medium, which was allowed to dry out. A drop of Hoechst stain (10 mg/mL; B2261; Sigma) prepared with sodium citrate (2.3%) was added on top of the blastocysts for 5 min, after which the slide was overlaid with a glass coverslip. The total number of cells was counted under a fluorescence microscope (λ 320) with the aid of image analysis software (MicroVision™, Danish Technology Institute, Taastrup, Denmark).

Experiment 1

Citrate in defined IVC medium. In 3 replicates, presumptive zygotes matured and fertilized following standard procedures were cultured in either M199 co-culture or in SOFaa (Table 1) with BSA (4 mg/mL; SOFaa-BSA) or PVA (3 mg/mL; SOFaa-PVA), with or without tri-sodium-citrate.

Experiment 2

Myo-inositol in defined vs undefined IVC medium. In Experiment 2a, presumptive zygotes matured and fertilized following standard procedures were cultured in SOFaac-BSA or in SOFaac-PVA with or without myo-inositol in a 2x2 factorial design (3 replicates). In all replicates, treatments were compared with the M199 co-culture system. In a separate replicate, the cell numbers of early and expanded/hatched blastocysts developing in M199 co-culture or in SOFaac-PVA with myo-inositol (i.e., SOFaaci) were compared.

In vivo survival of blastocysts developed in defined IVC medium. In Experiment 2b, Day 7 blastocysts produced in SOFaaci-PVA were shipped by air at 4 different days in 6-mL Falcon® tubes containing Hepes-buffered M199 with 3 mg/mL PVA at 38° C in a portable mini-incubator (MiniTüb GmbH, Tiefenbach, Germany). Morphologically good blastocysts were

selected and transferred nonsurgically within 6 h of shipment into recipient heifers on Day 7 of the estrous cycle (Day 0 = day of estrus). Heifers had been synchronized with 2 injections of cloprostenol (0.5 mg im; Estrumate®, Mallinckrodt Veterinary Ltd, Harefield, England) 11 days apart. Pregnancies were diagnosed per rectum on Days 48 and 60, and pregnant animals were allowed to go to term.

Experiments 3

Defined IVC vs undefined IVC. In Experiment 3a (4 replicates), presumptive zygotes matured and fertilized following standard procedures were cultured in either 1) M199 co-culture, 2) SOFaaci-PVA or 3) SOFaaci-CS. Embryo development was evaluated on Day 9 post insemination.

Stability of the SOFaaci-CS System. In Experiment 3b, running in 30 weekly replicates from May 1997 to March 1998, presumptive zygotes were matured and fertilized following standard procedures and then cultured for 8 d in SOFaaci-CS.

Experiments 4

High vs low serum concentrations vs BSA in IVF. In 3 replicates of Experiment 4a, COC's matured in standard M199-CS medium were randomly allocated into 3 groups for fertilization in TLP-medium containing either 1) the standard ~1% CS carried over from IVM at transfer of COC's (TLP-CS), 2) 10% CS (TLP-10% CS or 3) 6 mg/mL BSA (TLP-BSA). Each experimental group was inseminated with spermatozoa that had been washed in TLP with protein supplementation corresponding to the respective IVF-medium. After fertilization, presumptive zygotes were cultured for 8 d in M199 co-culture.

Defined IVM vs defined IVM-IVF vs undefined IVM-IVF. In Experiment 4b, immature oocytes were randomly allocated to 3 groups for maturation in either 1) standard M199-CS followed by 3 washes in the same medium and standard IVF in TLP-CS (undefined IVM-IVF); 2) M199 followed by 3 washes in M199-CS and standard IVF in TLP-CS (defined IVM); or 3) M199 followed by 3 washes in M199 and IVF in TLP (defined IVM-IVF). After fertilization, the presumptive zygotes were cultured in SOFaaci-CS. In the first replicate, 20 mature oocytes and 20 zygotes from each treatment group were randomly selected, fixed and stained in order to assess nuclear maturation and fertilization.

Experiment 5

Defined IVC vs defined IVP vs undefined IVP. In 5 replicates, immature COC's were randomly allocated into 3 groups: 1) undefined IVP - standard IVM, IVF and IVC in M199-CS, TLP-CS and SOFaaci-CS, respectively; 2) defined IVC - standard IVM and IVF as Group 1 followed by IVC in SOFaaci-PVA; and 3) defined IVP - IVM, IVF and IVC in M199, TLP and SOFaaci-PVA, respectively. In 2 of the replicates, the compact morulae and blastocysts were fixed at Day 7 after insemination and their cell numbers were counted.

Statistical Analysis

Cleavage and blastocyst rates per oocyte or per cleaved embryo are given as mean of replicates ± SD. Developmental data were assumed to be binomially distributed and were analyzed by logistic regression using a generalized linear model (Genmod procedure; 40). Treatment and replicate were included in the model as co-variates. Cell numbers (mean ±

SD) were compared using ANOVA-test (GLM-procedure; 41). Probabilities (P-values) of less than 0.05 were regarded as significant.

RESULTS

In the experimental groups maturing under defined conditions (i.e., in M199 with hCG, eCG and without serum), cumulus expansion was reduced compared with maturation under similar conditions but with serum supplementation of the medium. Serum and BSA also allowed cumulus cells still remaining on the zona pellucida after the vortexing procedure to plate out on the bottom of the culture well. Subsequently, patchy circular monolayers of cumulus cells (1/3 to 2/3 of the diameter to the well) formed within the IVC period. In contrast, in all the experimental groups cultured in defined medium without serum or BSA, monolayers were not formed. Instead, cumulus cells remained on the zona pellucida, forming rounded cells that adhered to cumulus cells of neighboring ova. Consequently, clusters of adhering ova were regularly observed in the culture dishes at Day 7 post insemination. This was most obvious if the zygotes had also been matured and fertilized in defined medium, as their cumulus cells were more resistant to vortexing than those of oocytes matured and fertilized in the presence of serum.

Experiment 1

A total of 401 presumptive zygotes was used in this experiment (Table 3). There was no effect of type of IVC medium on the cleavage rate, but both the percentages of blastocysts per oocyte and per cleaved embryo were affected by treatment ($P<0.01$). Neither replicate nor interaction between medium and replicate influenced the results significantly. Substituting BSA with PVA in the SOFaa medium reduced blastocyst development significantly ($P=0.01$). However, adding tri-sodium citrate to the defined SOFaa-medium supplemented with PVA increased blastocyst development to a level not significantly lower than in SOFaa with BSA. Embryos cultured in M199 co-culture developed better than in SOFaa-PVA with or without citrate.

Table 3. Effect of substituting BSA with PVA, with or without tri-sodium citrate, in SOFaa medium on embryo development in vitro[a]

Medium	Supple-mentation	Oocytes n	Cleaved / oocyte	Blastocysts / oocyte	Blastocysts / zygote
SOFaa	BSA	87	79 ± 1%	30 ± 14%[b]	37 ± 12%[b]
SOFaa	PVA	105	83 ± 5%	16 ± 6%[c]	19 ± 6%[c]
SOFaa	PVA + citrate	98	81 ± 6%	23 ± 5%[d]	28 ± 8%[d]
M199 co-culture	CS	111	83 ± 6%	36 ± 13%[be]	42 ± 12%[be]

[a]mean ± SD of 3 replicates. No difference between replicates or interactions between replicate and medium.

[bcde]Dissimilar superscripts within columns denote statistical differences: [bc]$P<0.01$; [de]$P<0.05$.

Experiment 2

In Experiment 2a, a total of 662 oocytes were allocated to the 5 treatment groups (Table 4). Cleavage rates were not influenced by treatment or interactions between treatment

and replicate, but differed between replicates (87, 74 and 74%, respectively; P=0.001). In SOFaac medium, blastocyst rates of oocytes and of cleaved embryos were affected both by macro-molecular supplements (BSA vs PVA, P<0.05) and by the presence of myo-inositol (P<0.01). Furthermore, there was an interaction between these 2 factors (P<0.02). Hence, the addition of myo-inositol to SOFaac-PVA increased blastocyst development significantly to the levels observed in undefined IVC medium groups, but it did not have any additional effect in SOFaac-BSA. In contrast, hatching was only affected by a macromolecular supplement (P<0.01) and not by the myo-inositol component. Hatching rates tended to be higher in this experiment in medium with BSA than without it. Replicate also influenced the proportion of hatching within the culture period (P=0.01). At Day 7 after insemination, blastocyst development was lower in M199 co-culture (25%) and in defined SOF-medium without myo-inositol (i.e., SOFaac-PVA; 22%) than in the the other SOF-media (37 to 41%; P<0.01). However, on Day 9 more blastocysts had developed in the M199 co-culture (Table 4). The average cell numbers on Day 7 of expanded/hatched blastocysts and early blastocysts were 113 ± 29 (n=23) and 77 ± 25 (n=27), respectively, in M199 and 134 ± 29 (n=54) and 70 ± 23 (n=27), respectively, in SOFaac supplemented with myo-inositol and PVA (M199 vs SOFaacI; P≥0.1).

Table 4. Effect of BSA, PVA and myo-inositol on embryo development in SOFaac[a]

Medium	Macro-molecule	Myo-inositol	Oocytes n	Cleaved / Oocyte	Day 9 blastocysts / oocyte	Day 9 blastocysts / cleaved	Hatched / total blastocysts
SOFaac	BSA	-	146	78 ± 7%	47 ± 3%[b]	60 ± 2%[b]	51 ± 10%[e]
SOFaac	BSA	+	135	77 ± 4%	43 ± 7%[b]	56 ± 7%[b]	47 ± 4%[e]
SOFaac	PVA	-	134	79 ± 6%	19 ± 3%[c]	23 ± 3%[c]	26 ± 13%[f]
SOFaac	PVA	+	132	76 ± 3%	42 ± 1%[b]	54 ± 3%[b]	34 ± 14%[ef]
M199 co-culture	CS		115	81 ± 5%	36 ± 7%[d]	45 ± 8%[d]	41 ± 15%[ef]

[a]Mean ± SD of 3 replicates. Significant differences between replicates (P<0.001) in respect to cleavage and hatching.
[bcde]Dissimilar superscripts within columns denote statistical differences ([bc]P<0.001, [cd]P<0.01, [ef]P<0.05).

In Experiment 2b, a total of 33 blastocysts was produced under defined conditions in SOFaacI-PVA medium following standard undefined IVM and IVF. Ten blastocysts of good morphology were selected and transferred to 10 recipients following air-shipment. Five recipients became pregnant and gave birth to 5 healthy Danish Holstein-Friesian calves, 3 female and 2 males weighing from 37 to 45 kg at birth.

Experiment 3

Following standard undefined IVM and IVF procedures in Experiment 3a, a total of 1,043 presumptive zygotes was randomly allocated into the 3 treatment groups: Defined IVC in SOFaacI or undefined IVC's in SOFaacI-CS or in M199 co-culture. Results are shown in Table 5. Embryo development tended to be lower in M199 co-culture than in SOFaacI-CS or SOFaacI-PVA with respect to cleavage rates (P<0.1) and lower in respect to blastocyst development (P<0.05). A similar trend was observed for hatching. Furthermore, embryos in

both SOFaaci media appeared to be different compared with embryos in M199 co-culture with respect to the following details: Compaction was generally more evident, blastocyst formation was initiated earlier in relation to time of fertilization, embryos were lighter in color, and the inner cell mass of blastocysts was more distinct. The acceleration of blastocyst development was especially evident for embryos cultured in SOFaaci-CS. In this medium, blastocysts were observed at Day 6 (i.e., approximately 1 d earlier than in M199 co-culture).

Table 5. Comparison of embryo development in standard M199 granulosa cell co-culture or in SOFaaci under defined (SOFaaci-PVA) or undefined (SOFaaci-CS) conditions[a]

Treatment	Oocytes *n*	Cleaved / oocyte	Day 9 blastocysts / oocyte	Day 9 blastocysts / zygote	Hatched per total blastocysts
SOFaaci - CS	361	86 ± 3%	42 ± 7%[b]	49 ± 8%[b]	69 ± 7%[b]
SOFaaci - PVA	332	83 ± 6%	42 ± 9%[b]	51 ± 9%[b]	60 ± 16%[bd]
M199 co-culture	350	78 ± 2%	32 ± 7%[c]	40 ± 9%[d]	50 ± 7%[d]

[a]Mean ± SD in 4 replicates. Overall embryo development differed between replicates ($P<0.05$), but no significant interactions between treatment and replicate were observed.
[bcd]Dissimilar superscripts within columns denote statistical differences ([bc]$P<0.01$; [bd]$P<0.05$).

In Experiment 3b, the average ± SD (range) cleavage rate and blastocyst development per oocyte and cleaved embryo in SOFaaci-CS over 30 weekly replicate cultures were 87 ± 4% (78 to 93%), 49 ± 5% (40 to 61%) and 57 ± 6% (45 to 68%), respectively. Cleavage rates in the first test quarter of 1997 (5 replicates in May and June) were significantly lower than in the other quarters (79 vs 87 to 90%, $P<0.001$), and replicate tended to influence cleavage ($P=0.06$). However, blastocyst development per oocyte did not vary significantly between replicate or season. The results are shown in Figure 1.

Experiment 4

In Experiment 4a, a total of 611 mature oocytes was allocated randomly into the 3 IVF-treatment groups: standard IVF in TLP-CS (n=203), IVF in TLP-BSA (n=212), or TLP-10% CS (n=196). Neither cleavage rates (84 ± 2, 82 ± 4 and 82 ± 2%, respectively), nor blastocyst rates (33 ± 5, 34 ± 9 and 33 ± 3%, respectively) differed significantly between treatments ($P>0.5$) following IVC in M199 co-culture. There was no influence of replicate or of treatment by replicate interaction on the results.

In Experiment 4b, a total of 814 COC's was subjected to 1 of 3 IVM-IVF treatments: undefined standard IVM-IVF, defined IVM - undefined IVF and defined IVM-IVF (Table 6). Treatment affected both cleavage and blastocyst development ($P<0.001$), but not hatching in the subsequent IVC in SOFaaci-CS medium. The presence or absence of serum during IVM did not have any obvious influence on nuclear maturation, as no differences were observed between treatment groups in the numbers of oocytes with a normal metaphase configuration (18 of 20, 18 of 19, and 14 of 16 successfully stained oocytes for undefined standard IVM-IVF, defined IVM–undefined IVF, and defined IVM-IVF groups, respectively). Normal fertilization characterized as the presence of (only) 2 morphologically normal pro-nuclei were found in 16 of 19, 15 of 19 and 9 of 16 ova successfully fixed and stained in undefined standard IVM-IVF, defined IVM–undefined IVF and defined IVM-IVF, respectively, thus

reflecting the in vitro development (Table 6). Three pro-nuclei were observed in 1 ovum from the standard treatment group. The rest of the stained ova were either in metaphase II or were nondeterminable (i.e., no visible nuclei or stained chromatin). No differences in sperm motility were observed 20 h after insemination (i.e., prior to vortexing of the presumptive zygotes) under defined conditions in TLP vs standard undefined conditions in TLP-CS medium.

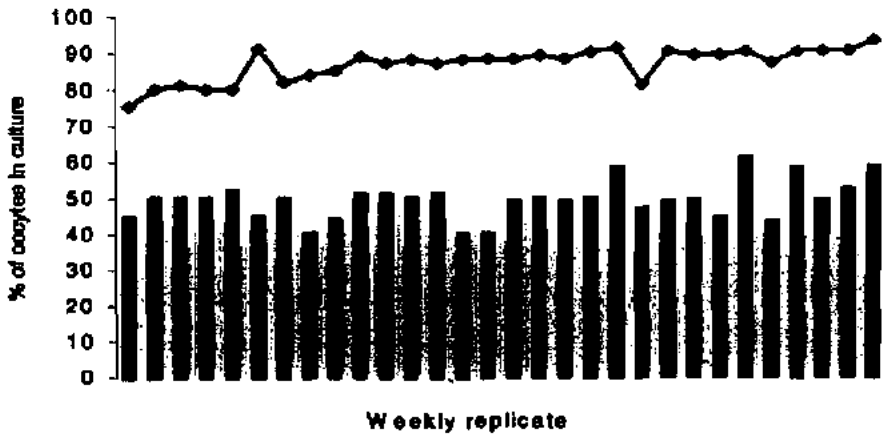

Figure 1. Embryo development in SOFaaci with 5% CS. A total of 30 weekly replicates within the period of May 1997 to March 1998 with 100 COC's per trial. The curve and columns illustrate cleavage and blastocyst rates, respectively.

Experiment 5

A total of 749 oocytes was allocated to the 3 treatment groups (Table 7). Except for hatching, embryo development was affected significantly ($P<0.001$) by treatment. As in Experiment 4b, the absence of serum during IVM-IVF reduced the cleavage rate. However, in the present experiment, subsequent development of the cleaved embryos under defined conditions was also negatively affected when compared with embryos that had been exposed to serum during IVM-IVF. No differences were observed in cleavage or blastocyst rates between embryos cultured with serum or under defined conditions when serum was present during IVM-IVF. Finally, no differences in the capability of blastocysts to hatch in vitro were noticed between any of the treatment groups. Hatching rates in this experiment were apparently higher than in Experiment 2 (Table 4), in which the hatching rates for blastocysts cultured under defined conditions tended to be lower than in the presence of BSA. As in Experiment 3a, blastocysts were observed to develop faster in serum. This was reflected in the cell numbers of compact morulae and blastocysts at Day 7. The overall average cell numbers of Day 7 compact morulae and blastocysts produced in 1) undefined IVM, IVF and IVC media; 2) undefined IVM and IVF media but defined IVC; and 3) defined IVM, IVF and IVC media were 1) 117 ± 48^{a} (n=53; all blastocysts), 2) 105 ± 33^{b} (n=31, 28

blastocysts) and 3) 89 ± 29[c] (n=41, 29 blastocysts) , respectively ([ac]P<0.01;[bc]P=0.06). However, no statistical differences were observed between treatments when embryo stages were included in the statistical analysis (P=0.25), only between embryo stages (P<0.001). The overall cell numbers of Day 7 hatched or expanded blastocysts (n=52), mid-blastocysts (n=38) and early blastocysts or compact morulae (n=27) were 134 ± 33[a], 95 ± 26[b], 76 ± 22[c] ([abc]P<0.05), respectively.

Table 6. Comparison of embryo development following maturation and fertilization in defined media vs undefined medium with serum[a]

IVM-IVF treatment	Oocytes n	Cleaved / oocyte	Day 7 blastocysts / oocyte	Day 9 blastocysts / oocyte	zygote	Hatched / blastocysts
Defined IVM Undefined IVF	269	83 ± 3%[b]	49 ± 8%[b]	51 ± 8%[b]	61 ± 5%	62 ± 6%
Defined IVM-IVF	278	55 ± 3%[c]	33 ± 3%[c]	32 ± 5%[c]	57 ± 6%	63 ± 13%
Undefined IVM-IVF	267	90 ± 1%[bd]	57 ± 6%[bd]	56 ± 3%[b]	62 ± 2%	64 ± 17%

[a]Mean ± SD of 3 replicates. Significant differences between replicates were observed with respect to blastocyst development (P=0.05), and significant interactions between replicate and treatment group were also observed for blastocysts at Day 7 and for hatching blastocysts at Day 9 (P<0.05).
[bcd]Dissimilar superscripts within columns denote statistical differences ([bc]P<0.001; [bd]P<0.01).

Table 7. Comparison of embryo development following IVM, IVF and IVC in defined media or in standard undefined media with serum[a].

Treatment	Serum in IVM/IVF	Serum in IVC	Oocytes n	Cleaved / oocyte	Day 7 blastocysts / oocyte	Day 9 Blastocysts / oocyte	Day 9 Blastocysts / zygote	Hatched / total blastocysts
1	+	+	255	84 ± 4%[a]	48 ± 8%[b]	48 ± 2%[b]	57 ± 4%[b]	39 ± 16%
2	+	-	207	89 ± 1%[a]	44 ± 7%[b]	46 ± 6%[b]	52 ± 7%[b]	55 ± 10%
3	-	-	287	54 ± 7%[b]	15 ± 3%[c]	19 ± 4%[c]	37 ± 6%[c]	51 ± 10%

[a]Mean ± SD of 3 replicates. Differences between replicates were observed (P<0.01), but no significant interactions between replicate and treatment group were present.
[bc]Dissimilar superscripts within columns denote statistical differences ([bc]P<0.001).

DISCUSSION

The results of the present study demonstrate that immature bovine oocytes are able to mature, fertilize and develop to the blastocyst stage in defined media without serum or BSA, thus confirming earlier findings (11, 29). Serum-proteins could be excluded from in vitro culture media with only marginal negative influence on blastocyst development compared to

control conditions when sodium citrate and myo-inositol were added to the SOFaa medium. However, omission of serum or BSA from both IVM and IVF reduced fertilization to approximately two-thirds that of the controls (Tables 6 and 7). Moreover, continued culture under defined conditions resulted in a further reduction in embryonic development compared with that of zygotes matured and fertilized in defined media but cultured with serum. Exclusion of serum from the maturation medium had only a lesser influence on the subsequent fertilization and cleavage of embryos, as long as serum was present in low concentrations during fertilization. The birth of 5 healthy calves following transfer of 10 blastocysts cultured in SOFaaci-PVA confirms that the defined culture system supports development of viable embryos.

The embryotrophic effect of sodium citrate as observed in the present study has been reported earlier (20, 30), and the concentration used is within the optimal range for in vitro development in rabbit embryo culture (20). Citrate stimulates fatty acid synthesis (19) and is a chelator of metal ions (e.g., Ca^{2+}), a feature that may be of importance for maintaining junctional integrity and thus of importance for compaction and blastocoel formation (20). In the present study, the addition of tri-sodium citrate to defined SOFaa with PVA enhanced blastocyst development. The effect of adding citrate to SOFaa with BSA was not investigated. The BSA preparation had been tested earlier for its embryotrophic properties (25), which is likely to reflect a significant citrate contamination of this specific preparation (20). However, it is possible that addition of citrate to SOFaa with the present BSA further enhanced blastocyst development, since blastocyst development was higher in SOFaac-BSA in Experiment 4a than in the same medium without citrate in Experiment 3 (47 ± 3 vs 30 ± 14%; Tables 3 and 4).

The subsequent addition of myo-inositol to the SOFaa with citrate and PVA (i.e., SOFaac-PVA) further improved blastocyst development to a degree that did not differ from that of culture conditions including BSA, serum and/or granulosa cells. Myo-inositol increased blastocyst development in defined medium two-fold, while no effect was seen in SOFaac with BSA (Table 4). The effect on hatching was not evident and tended to be lower with higher variability than in controls. This was particularly manifest in Experiment 2, in which the effect of myo-inositol in SOFaac with PVA or BSA (Table 4) was studied, while less evident in Experiment 3 comparing embryo development in SOFaaci-PVA vs SOFaaci-CS, where hatching rates were high in both groups (≥60%; Table 5). We do not have any explanation for this variability, but factors other than myo-inositol (and citrate) present in BSA and/or serum are likely to influence hatching in vitro. Furthermore, serum has been reported to decrease replicate variation in embryo development (48). We are at present comparing the long-term stability (e.g., blastocyst hatching) under chemically defined vs undefined conditions in order to clarify the significance of the observed variability. Myo-inositol is present in a range of complex media, and is often categorized as a vitamin (42). It is also likely to be present in commercial BSA-preparations in minute concentrations but sufficient for biological action (C.P. Downes, personal communication), thus explaining the lack of an additional effect in medium with BSA. Myo-inositol or its metabolites are essential components in cellular signaling (for review, see 10). Inositol-1,4,5-triphosphate has second messenger functions, and some metabolites may have a direct mitogenic effect (10). Specifically, myo-inositol is associated with intracellular calcium release at fertilization (for review, see 31), and it behaves as an osmolyte together with glycine, providing substantial protection of rabbit embryos cultured in medium with high sodium chloride concentration (33). Furthermore, in sea urchin embryos, myo-inositol has been shown to be essential for proper timing of the first cell cycle by its participation in the phosphoinositide-signaling

pathway (5). Finally, embryotrophic properties of batches of human serum have been correlated with high concentration of myo-inositol (8).

In the present study, blastocysts developed at a higher frequency in SOFaaci culture with or without serum than in M199 granulosa cell co-culture system (53), with similar cell numbers of the respective embryo developmental stages and similar or higher blastocyst hatching rates (Tables 4 and 5). Using SOFaaci with 5% CS, we consistently obtained around 50% blastocysts of oocytes in culture during the 11-mo test period (49 ± 5%; Figure 1). This overall efficiency was slightly higher than we have obtained earlier in our laboratory using M199 granulosa cell co-culture (47%; 53). However, most importantly, embryos produced in SOFaaci medium - defined or undefined - appeared morphologically more like in vivo embryos, considering their lighter color, tighter compaction and the integrity of the inner cell mass (55). Blastocysts formed earlier in SOFaaci particularly with the inclusion of serum compared to M199 co-culture. Others have reported similar observations (9). It is well known that serum changes the pattern of blastulation by inducing blastocoel formation (37, 54, 57). Very high serum concentrations in basic SOF without amino acid supplementation result in premature blastulation of ovine embryos (49, 56). Bovine embryos have also been observed to blastulate earlier, without corresponding cell proliferation, when serum is added to SOFaa-medium containing BSA (48, 52). However, in the present study, very tight compaction at the morula stage preceded blastulation in SOFaaci with or without serum, although the development of blastocysts was slower without serum (i.e., in SOFaaci-PVA). The overall cell number of Day 7 compact morulae and blastocysts tended to be lower under defined conditions, but cell numbers of the respective embryo stages did not differ between treatments, indicating that embryo quality were similar with or without serum. Similar observations were made when comparing blastocyst development in SOFaaci and M199 co-culture. The faster development in SOFaaci should, therefore, be regarded as a sign of improved culture conditions, as the accelerated blastocyst formation was accompanied with cell proliferation.

In addition, the SOFaaci system is a simple IVP-system, as embryos may be left in culture for a period of 9 d without changing medium or preparation of somatic cells for co-culture. For routine IVP, oocytes and embryos are transferred between IVM, IVF and IVC without intermediate washes, making the system even simpler. However, for sanitary reasons, washes are desirable and required (27). The outgrow of a patchy granulosa cell monolayer from cumulus cells remaining on the zona pellucida in SOFaaci with 5% CS may also constitute a sanitary risk (2, 44). However, careful cleaning of zygotes after IVF and culture in totally cell-free modified SOFaa is possible without affecting the culture result (7). Under defined SOFaaci conditions the remaining cumulus cells stayed at the zona pellucida, and noticeable proliferation was not observed. It cannot be deduced from the present results if these cells have a possible embryotrophic and conditioning effect on the medium (34).

Nevertheless, a calving rate of 50% of the transferred embryos suggests that the IVP-blastocysts cultured in defined SOFaaci-medium were of a quality similar to that of embryos produced under undefined culture systems (22).

Omission of serum from the IVM-medium did not affect nuclear maturation of oocytes or the subsequent development of cleaved embryos, which is similar to that of other reports (3, 11, 13, 38). However, fertilization expressed as the cleavage rate of oocytes matured in the absence of serum was slightly decreased. In contrast, a large significant reduction in the fertilization rate (expressed as ratio of ova with 2 normal pronuclei or cleavage rate following insemination) was observed, when serum or BSA were absent from both maturation and

fertilization media (including sperm preparation). Zygotic development, expressed as number of blastocysts per cleaved embryo, was only affected if the subsequent culture was performed in the absence of serum. The relative low fertilization rate in this and another study (11) may be explained by the fertilization medium, which was modified from Tyrode's medium. Other research groups (30, 39, 45) have found that fertilization was only slightly reduced under defined culture conditions using modified Brackett and Oliphant's medium (BO-medium; 6), but still no more than 60% of oocytes cleaved. Keskintepe et al. (30) found specifically that BO-medium was superior to TALP in which BSA was substituted with PVA. Furthermore, the inclusion of PVA in BO-medium has been found to improve fertilization (30, 45). The present study indicates that exposure of the mature oocyte and/or 1-cell embryo to component(s) in serum is of utmost importance both for optimal fertilization and development of fertilized embryos under our conditions. Eckert and Niemann (11) concluded in a study on defined IVP of bovine embryos that serum-proteins were required for normal pronuclei formation but not for cleavage and subsequent embryo development. In a recent study from our laboratory (21), we observed that 2 min of exposure to serum compared to no exposure prior to IVF of oocytes matured, fertilized and cultured in defined media improved not only cleavage (63 vs 81%) but also blastocyst development of the cleaved embryos (33 vs 48%) to levels not significantly different from embryos exposed to serum throughout the respective culture steps.

In conclusion, the present results show that tri-sodium citrate and especially myo-inositol improved the SOFaa-medium, thus more than 45% blastocysts developed from immature oocytes without the conventional supplementation with serum or BSA. However, exposure to serum during the IVM and/or IVF periods was essential for acceptable fertilization rates under the present conditions (i.e., >75% cleaved embryos) and for subsequent high blastocyst development (i.e., >50% blastocysts of cleaved embryos) under defined conditions in SOFaaci. Hence, 19% of the inseminated oocytes would develop to the blastocyst stage under completely defined IVP-conditions. The SOFaaci culture system, with or without serum-proteins, is simple to work with: no change of medium or preparation of co-culture is needed. Finally, morphology and in vivo viability of embryos produced in SOFaaci suggest that they are of a quality similar to that of in vivo embryos. However, larger field trials are needed to verify the in vivo viability of embryos produced under completely defined conditions.

REFERENCES

1. Aoyagi Y, Fukui Y, Iwazumi Y, Urakawa M, Ono H. Effects of culture systems on development of in vitro fertilized bovine ova into blastocysts. Theriogenology 1990; 34:749-760.
2. Avery B, Greve T, Rønsholt L, Bøtner A. Virus screening of a bovine in vitro embryo production system. Vet Rec 1993; 132:660.
3. Avery B, Bavister BD, Greve T. Development of bovine oocytes in vitro matured in a chemically defined protein-free medium, supplemented with different amino acid formulations. Theriogenology 1998; 49:306 abstr.
4. Bavister BD. Culture of preimplantation embryos: facts and artifacts. Hum Reprod Update 1995; 1:91-148.
5. Becchetti A, Whitaker M. Lithium blocks cell cycle transitions in the first cell cycle of sea urchin embryos, an effect rescued by myo-inositol. Development 1997; 124:1099-1107.
6. Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa in vitro. Biol Reprod 1975; 12:260-274.

7. Carolan C, Lonergan P, Van Langendonckt A, Mermillod P. Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in vitro. Theriogenology 1995; 43: 1115-1128.
8. Chiu TT, Tam PP. A correlation of the outcome of clinical in vitro fertilization with the inositol content and embryotrophic properties of human serum. J Assist Reprod Genet 1992; 9:524-530.
9. Donnay I, Van Langendonckt A, Anquier P, Grisart B, Vansteenbrugge A. Massip A, Dessy F. Effects of co-culture and embryo number on the in vitro development of bovine embryos. Theriogenology 1997;47:1549-1561.
10. Downes CP, MacPhee CH. Myo-inositol metabolites as cellular signals. Eur J Biochem 1990; 193:1-18.
11. Eckert J, Niemann H. In vitro maturation, fertilization and culture to blastocysts of bovine oocytes in protein-free media. Theriogenology 1995; 43:1211-1225.
12. Eckert J, Niemann H. Effect of platelet-derived growth factor (PDGF) on the in vitro-production of bovine embryos in protein-free media. Theriogenology 1996; 46:307-320.
13. Ectors FJ, Fontes RS, Thonon F, Delval A, Figueiredo JR, Beckers J-F, Ectors F. Effect of non-protein medium during in vitro maturation on in vitro development of bovine embryos. Theriogenology 1992; 37:206 abstr.
14. Eyestone WH, First NL. Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. J Reprod Fertil 1989; 85:715-720.
15. Flood LP, Shirley B. Reduction of embryo toxicity by protein in embryo culture media. Mol Reprod Dev 1991; 30:226-231.
16. Fukui Y, McGowan LT, James RW, Pugh PA, Tervit HR. Factors affecting the in-vitro development to blastocysts of bovine oocytes matured and fertilized in vitro. J Reprod Fertil 1991; 92:125-131.
17. Gardner DK. Mammalian embryo culture in the absence or presence of serum or somatic cell support. Cell Biol Int 1994; 18:1163-1179.
18. Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins and culturing embryos in groups stimulate development. Biol Reprod 1994; 50:390-400.
19. Goodridge AG. Regulation of fatty acid synthesis in isolated hepatocytes. Evidence for a physiological role for long chain fatty acyl coenzyme A and citrate. J Biol Chem 1973; 248:4318-4326.
20. Gray CW, Morgan PM, Kane MT. Purification of embryotrophic factor from commercial bovine serum albumin and its identification as citrate. J Reprod Fertil 1992; 94:471-480.
21. Holm P, Booth PJ, Callesen H. Brief exposure of in vitro matured bovine oocytes to serum prior to fertilization in defined conditions enhances embryo development. Theriogenology 1999; 51:320 abstr.
22. Holm P, Callesen H. In vivo versus in vitro produced embryos: Similarities and differences relevant for practical application. Reprod Nutr Dev 1998; 38:579-594.
23. Holm P, Booth PJ, Vajta G, Callesen H. A protein-free SOF system supplemented with amino acids, sodium citrate and myo-inositol for bovine embryo culture. Proc 13th Sci Meeting Europ Emb Transf Assoc 1997;158 abstr.
24. Holm P, Irvine BJ, Armstrong DT, Seamark RF. Effect of oviduct epithelial cells on the fertilization and development of sheep oocytes in vitro. Anim Reprod Sci 1994; 36:227-241.
25. Holm P, Vajta G, Greve T, Callesen H. Effect of different protein sources on the in vitro development of bovine in vitro zygotes. Proc 11th Sci Meeting Europ Emb Transf Assoc 1995;190 abstr.

26. Holm P, Walker SK, Seamark RF. Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. J Reprod Fertil 1996; 107:173-181.
27. International Embryo Transfer Society. Manual of the International Embryo Transfer Society. A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures. Stringfellow DA, Seidel SM (eds). International Embryo Transfer Society, 1998.
28. Kane MT, Headon DR. The role of commercial bovine serum albumin preparations in the culture of one-cell rabbit embryos to blastocysts. J Reprod Fertil 1980; 60:469-475.
29. Keskintepe L, Brackett BG. In vitro developmental competence of in vitro-matured bovine oocytes fertilized and cultured in completely defined media. Biol Reprod 1996; 55:333-339.
30. Keskintepe L, Brunley CA, Brackett BG. Production of viable bovine blastocysts in defined in vitro culture conditions. Biol Reprod 1995; 52:1410-1417.
31. Kline D. Activation of the mouse egg. Theriogenology 1996; 45:81-90.
32. Lee ES, Fukui Y. Effect of various growth factors in a defined culture medium on in vitro development of bovine embryos matured and fertilized in vitro. Theriogenology 1995; 44:71-83.
33. Li J, Foote RH. Effect of inositol and glycine with increasing sodium chloride and constant osmolality on development of rabbit embryos. J Assist Reprod Genet 1995; 12:141-146.
34. Mermillod P, Vansteenbrugge A, Wils C, Mourmeaux J-L, Massip A, Dessy F. Characterization of the embryotrophic activity of exogenous protein-free oviduct conditioned medium used in culture of cattle embryos. Biol Reprod 1993; 49:582-587.
35. Parrish JJ, Susko-Parrish JL, Liebfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986; 25:591-600.
36. Pinyopummintr T, Bavister BD. In vitro-matured/in vitro-fertilized bovine oocytes can develop to morulae/blastocysts in chemically defined protein-free culture media. Biol Reprod 1991;45, 736-742.
37. Pinyopummintr T, Bavister BD. Development of bovine embryos in a cell-free culture medium: effects of type of serum, timing of its inclusion and heat inactivation. Theriogenology 1994; 41:1241-1249.
38. Saeki K, Hoshi M, Leibfried-Rutledge ML, First NL. In vitro fertilization and development of bovine oocytes matured in a protein-free medium. Biol Reprod 1991; 44:256-260.
39. Saeki K, Nagao Y, Hoshi M, Nagai M. Effects of heparin, sperm concentration and bull variation on in vitro fertilization of bovine oocytes in a protein-free medium. Theriogenology 1995; 43:751-759.
40. SAS Technical Report P-243, SAS/STAT Software:, The GENMOD Procedure. Release 6.09. Cary NC: SAS Institute Inc, 1993.
41. SAS/STAT™ User's Guide, Release 6.03. Cary NC: SAS Institute Inc, 1993; 549-641.
42. Sigma Chemical Company Ltd. Sigma Cell Culture Catalogue and Price List. St Louis MO, 1993.
43. Sinclair KD, McEvoy TG, Carolan C, Maxfield EK, Maltin CA, Young LE, Wilmut I, Robinson JJ, Broadbent PJ. Conceptus growth and development following in vitro culture of ovine embryos in media supplemented with bovine sera. Theriogenology 1998; 49:218 abstr.
44. Springfellow DA, Wrathall AE. Epidemiological implications of the production and transfer of IVF embryos. Theriogenology 1995; 43:89-96.

45. Tajik P, Wang W-H, Okuda K, Niwa K. In vitro fertilization of bovine oocytes in a chemically defined, protein-free medium varying the bicarbonate concentration. Biol Reprod 1994; 1231-1237.
46. Takahashi Y, First NL. In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992; 37:963-978.
47. Tervit HR, Whittingham DG, Rowson LEA. Successful culture of sheep and cattle ova. J Reprod Fertil 1972; 30:177-179.
48. Thompson JG, Allen NW, McGowan LT, Bell ACS, Lambert MG, Tervit HR. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. Theriogenology 1998; 41:1239-1249.
49. Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by the culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod 1995; 53:1385-1391.
50. Thompson JG, Duganzich D. Analysis of culture systems for bovine in vitro embryo production reported in abstracts of the proceedings of the international embryo transfer society (1991-1995). Theriogenology 1996; 45:195 abstr.
51. Trachte E, Stringfellow D, Riddell K, Galik P, Riddell M Jr, Wright J. Washing and trypsin treatment of in vitro derived bovine embryos exposed to bovine viral diarrhea virus. Theriogenology 1998; 50:717-716.
52. Tricoire H, Touzé J-L, Mermillod P. Effect of fetal calf serum on the quality of in vitro produced cattle embryos. Theriogenology 1999; 51:257 abstr.
53. Vajta G, Holm P, Callesen H, Greve T. Overall efficiency of in-vitro embryo production and vitrification in cattle. Theriogenology 1996; 45:683-689.
54. Van Langendonckt A, Donnay I, Schuurbiers N, Auquier P, Carolan C, Massip A, Dessy F. Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J Reprod Fertil 1997; 109:87-93.
55. Van Soom A, Bols PEJ, Vanroose G, de Kruif A. A comparison between in vivo and in vitro produced bovine embryos: Scientific and practical implications. Proc. 12th Int Conf Anim Reprod Artif Insem 1992; 3; 1365-1367.
56. Walker SK, Heard TM, Seamark RF. In-vitro culture of sheep embryos without co-culture: successes and perspectives. Theriogenology 1992; 37:111-126.
57. Yoshioka K, Othman AM, Taniguchi T, Yamanaka H, Sekikawa K. Differential patterns of blastulation in bovine morulae cultured in synthetic oviduct fluid medium containing FCS or BSA. Theriogenology 1997; 48:997-1008.

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IN VITRO DEVELOPMENT OF BOVINE ONE-CELL EMBRYOS: INFLUENCE OF GLUCOSE, LACTATE, PYRUVATE, AMINO ACIDS AND VITAMINS

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ABSTRACT

To elucidate the effect of nutrient substrates on embryo development, in vitro fertilized bovine one-cell embryos were cultured in a medium similar to synthetic oviduct fluid (SOF) but without glucose and containing 3.3 mM lactate, 0.3 mM pyruvate and 3 mg/ml bovine serum albumin (BSA) at 39°C in 5% CO_2 in air. Results indicated that addition of glucose was not only unnecessary, but it also had a deleterious effect on embryo development to the morula stage. Lactate supported embryo development up to the morula stage as well as pyruvate. Supplementation with 20 amino acids contained in basal medium Eagle's (BME) and minimum essential medium (MEM) improved development to the morula stage dramatically and increased the cell number compared with that of the controls. Addition of the vitamins from MEM to SOF had no beneficial effect. The SOF with amino acids did not increase the frequency of blastocysts 7 days after in-vitro fertilization but did increase the total number of cells compared with that of the controls. Frequency of blastocysts at Day 7 in SOF with amino acids was equivalent to that of co-culture although the total cell number was lower. These results demonstrate that a semi-chemically defined medium can successfully support the development of bovine embryos to the morula stage to a limited extent, but the medium lacks some nutrients or growth factors to fully support development through the blastocyst stage.

Key words: bovine embryo development, glucose, lactate, pyruvate, amino acid, vitamin

INTRODUCTION

Bovine embryos have been successfully cultured from the eight-cell to the blastocyst stage, while the culture of one-cell embryos to blastocysts has proved to be much more difficult (1) due to a developmental block that occurs in vitro at the eight-cell stage (2-4). Progress in embryo co-culture systems has made it possible to culture one-cell bovine embryos up to the blastocyst stage using various somatic cells in co-culture (3-6) or in conditioned medium (7). These co-culture systems or conditioned medium can be used to culture bovine embryos derived from in-vitro fertilization (5,6), nuclear transfer

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(8) and gene injection (Hoshi, M. et al., unpublished) to a stage of development at which they can be transferred into recipient cattle. The somatic cells used as co-culture may produce unknown embryo growth promoting factor(s) and/or delete embryo toxic factor(s) from a basic medium (7), making it difficult to define the exact requirements for embryo culture and development and/or preventing a clear understanding of embryo metabolism and development independent of other cell types. A successful embryo culture system using somatic cell-free medium is needed for the in-vitro study of embryo development. Such a system has been developed by Tervit et al. (9) based on components of sheep oviduct fluid and also Rosenkrans et al. (10). The efficiency of synthetic oviduct fluid (SOF; 9) for in-vitro development of bovine and ovine one-cell embryos was recently confirmed by several workers by adding human serum to SOF instead of bovine serum albumin (BSA; 11-13). However, another recent trial with the original SOF containing BSA failed to support the normal development of one-cell bovine embryos to the morula stage, even with reduced oxygen tension, and suggested a requirement for further refinement in the culture conditions (14). Human serum may contain unknown nutrients and embryotrophic factors. Completely chemically-defined media should be employed for the in-vitro study of embryonic nutrient requirement and utilization.

We report here experiments designed to test the hypotheses that glucose, lactate, pyruvate, amino acids, vitamins and bovine serum albumin are beneficial components in the culture of bovine embryos from one cell to morula and blastocyst stages.

MATERIALS AND METHODS

In Vitro Fertilization of Ovarian Oocytes

Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (1 to 5 mm in diameter) on bovine ovaries obtained from a slaughterhouse. They were washed three times with HEPES-buffered Tyrode's medium (TALP-HEPES; 15) supplemented with 3 mg/ml BSA (Fraction V, Sigma Chemical Co., St. Louis, MO); 0.2 mM sodium pyruvate; and 25 µg/ml of gentamicin sulfate (Sigma). Only oocytes with an intact, unexpanded cumulus and evenly granulated cytoplasm (16) were then cultured for 23 to 25 hours with TCM-199 supplemented with 10% heat-treated fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY); 0.5 µg/ml of FSH (NIH-oFSH); 5 µg/ml of LH (NIH-oLH); 1 µg/ml of estradiol-17β (Sigma); 0.2 mM sodium pyruvate; and 25 µg/ml of gentamicin sulfate (17). Maturation cultures as well as all the other cultures were maintained at 39°C in humidified air with 5% CO_2. In-vitro fertilization was performed using frozen semen from one ejaculate from one bull since the frequency of in-vitro fertilization and its time course had been well characterized in preliminary studies (semen donated by American Breeders Service, DeForest, WI). In these preliminary experiments, fertilization resulted in nearly 100% sperm penetration of oocytes and approximately 85% normal fertilization (two pronuclei). The frozen-thawed semen was diluted with modified Brackett and Oliphant isotonic medium (18) without BSA but with 1.7 µg/ml phenol-red and 25 µg/ml of gentamicin sulfate (BO medium). The sperm were then washed twice by centrifugation at 500 x g for 5 minutes. The COCs were coincubated for about 20 hours with spermatozoa (5 x 10^6 cells/ml) in BO medium supplemented with 2.5 mM theophylline and 3 mg/ml fatty-acids free BSA (Sigma) at 39°C in humidified air with 5% CO_2 and high humidity.

In Vitro Culture of One-Cell Embryos

In all experiments except Experiment 3, one-cell embryos were stripped of cumulus cells after coincubation with spermatozoa by vortexing in TALP-HEPES (17). The oocytes were then washed three times with each culture medium, and incubated in 100 µl-droplets covered with paraffin oil for 4 or 6 days under the same conditions as for maturation and fertilization. The formulation of modified synthetic oviduct fluid (mSOF) is shown in Table 1. Components of mSOF were similar to those of SOF medium (9), except for the amount of BSA added which was decreased to fall in line with most culture systems currently used and save on buffering capacity of the medium. A premixed solution of basal medium Eagles (BME) essential amino acids (Sigma #B6766); minimum essential medium (MEM) nonessential amino acids (Sigma #M7145); and 1.0 mM glutamine was added to mSOF in Experiments 3 and 4. A mixture of MEM vitamins (GIBCO #320-1120AG) was also used in Experiment 3. The osmolarity of the mSOF with supplements, but without BSA, was 270 to 280 mOsmols. The average pH of mSOF was 7.2 to 7.3. In some experiments, one-cell embryos were also co-cultured with bovine oviductal cells in TCM-199 supplemented with 10% FCS and 25 µg/ml gentamicin sulfate (TCM-FCS) (7).

Table 1. Composition of modified synthetic oviduct fluid (mSOF)

Component	Concentration
NaCl	107.70 mM
KCl	7.16
KH_2PO_4	1.19
$CaCl_2$	1.71
$MgCl_2$	0.49
$NaHCO_3$	25.07
Sodium lactate	3.30
Sodium pyruvate	0.30
BSA[a]	3.0 mg/ml
Phenol red	1.3 µg/ml
Gentamicin sulfate	50.0 µg/ml

a Crystallized and lyophilized; the osmolarity and pH of mSOF with or without supplements (see text) were 270 to 280 mOsmoles and 7.2 to 7.3, respectively.

Evaluation of Embryonic Development and Counting of Total Cell Number

Two days (approximately 44 hours) after in-vitro fertilization, initial cleavage rates were determined by examining the embryos under a stereomicroscope. Five days (122 to 124 hours) after in-vitro fertilization, all of the embryos except those with fewer than eight cells were mounted on a slide and fixed with a mixture of acetic acid and ethanol (1:3). After staining with 0.1% aceto-orcein, total cell number was counted under a phase-contrast microscope. Embryos which had ≥16 cells were defined as morula.

In separate experiments, all of the blastocysts obtained after 6 days of in-vitro culture (173 to 175 hours after oocyte insemination) were air-dried and their cell numbers were counted. In this procedure, blastocysts were treated with a hypotonic solution (0.9% sodium-citrate supplemented with 0.3% FCS) and were set with fixatives I (methanol:acetic acid:distilled water = 10:3:7) and II (methanol:acetic acid = 3:1). After

staining with 5% Giemsa solution, the total cell number, including metaphase plates but excluding pyknotic nuclei, were counted under a bright-field microscope.

Effect of [Glucose] on Development to Morulae (Experiment 1)

To determine the effect of glucose on the development of one-cell embryos beyond the 8- to 16-cell stage, one-cell embryos were cultured for 4 days in mSOF supplemented with various levels of glucose (0, 0.56, 1.5 and 5.56 mM). One-cell embryos were also co-cultured with oviductal cells. The embryos' initial cleavage rate, subsequent development to the morula stage and total cell number per morulae were recorded.

Effect of Metabolic Substrates on Development to Morulae (Experiment 2)

The necessity of lactate and pyruvate for early embryonic development was examined. One-cell embryos were cultured for 4 days in mSOF without lactate and/or pyruvate in the presence and absence of glucose (0.56 mM). The initial cleavage, development to the morula stage and total cell number in the morulae were determined.

Effects of Amino Acids and Vitamins on Development (Experiments 3a and 3b)

The effects of media supplementation with amino acids and vitamins on the development of one-cell embryos to the morula (Experiment 3a) and blastocyst stages (Experiment 3b) were studied. In these experiments, at about 20 hours after insemination, in-vitro fertilized eggs were transferred to each culture medium without removal of cumulus cells. The one-cell embryos were stripped of cumulus cells at 44 hours after fertilization by pipetting and were then further cultured for an additional 3 (Experiment 3a) or 5 days (Experiment 3b) in fresh medium without cumulus cells. Embryos were fixed at Day 5 or 7 (Day 0 = day of in-vitro insemination), and the percentages of morulae and blastocysts and their total cell numbers were recorded.

Effect of Protein in SOF and Glucose Added Post-compaction on Development to Blastocysts (Experiment 4)

To examine the effect of BSA and the addition of glucose through Days 5 to 7, one-cell embryos were cultured in mSOF supplemented with amino acids and 3 mg/ml BSA or 1 mg/ml polyvinylalcohol (PVA) (polyvinyl alcohol average molecular weight 10,000; Sigma) for 4 days. Five days after insemination, they were transferred to fresh medium with or without 5.56 mM glucose and were cultured for another 2 days. In this experiment, one-cell embryos were also co-cultured with oviductal cells for 6 days, and the frequency of blastocysts and total number of cells in the blastocysts were recorded.

Statistical Analysis

Differences in initial cleavage rates and percentages of one-cell embryos developing to the morula or blastocyst stage were analyzed by the Chi-square test. The total number of cells in morulae and blastocysts were subjected to logarithmic transformation, and were then assigned for ANOVA analysis. After ANOVA revealed a significant treatment effect, the treatments were compared by Duncan's multiple range test. In Experiments 1 and 4, the difference in the mean number of cells between each treatment and co-culture was analyzed by the Student's t-test.

RESULTS

Effect of [Glucose] on Development to Morulae (Experiment 1)

Although addition of glucose at any of the levels tested did not affect the initial cleavage rate, 5.56 mM glucose had a deleterious effect on the development of one-cell embryos to the morula stage, as shown in Table 2. In general, the total number of cells in morulae developed in mSOF was lower than that of contemporary embryos co-cultured with oviduct cells (Table 2).

Table 2. Effect of glucose on the development of one-cell embryos to the morula stage (Experiment 1)[a]

Glucose concentration (mM)	No. of oocytes inseminated	No. cleaved at Day 2 (%)	No. of morula at Day 5 (%)	Total no. of cells in the morulae	
				Mean±SD	Range
Modified synthetic oviduct fluid (mSOF)					
0	160	130 (81.3)	37 (23.1)b,d	24.6±9.4	16-58
0.56	127	97 (76.4)	29 (22.8)b,d	24.2±7.9	16-48
1.5	131	104 (79.4)	22 (16.8)b	25.3±9.0	16-48
5.56	101	81 (80.2)	6 (5.9)c	19.0±2.1	16-22
Co-culture with oviductal tissue in TCM199 + 10% FCS					
	122	97 (79.5)	41 (33.6)d	30.8+14.5[b]	16-67

a Day 0 = day of in-vitro insemination. Data are pooled from three to four replications.
b-d Values in the same column with different superscripts are different ($P<0.05$).

Effect of Metabolic Substrates on Development to Morulae (Experiment 2)

In the absence of glucose, when lactate and pyruvate were deleted from mSOF, both the initial cleavage rate and the percentage of morulae formation decreased ($P<0.05$), as shown in Table 3. Deletion of pyruvate with lactate present had no deleterious effect on frequency of morulae, but development to the morulae stage tended to decrease when lactate was deleted.

In the presence of 0.56 mM glucose, deletion of lactate or of both lactate and pyruvate caused a marked reduction in the initial cleavage rate while frequency of morula at Day 5 decreased only in the absence of both lactate and pyruvate. In medium from which only lactate was deleted, the cleavage rate decreased but the frequency of morulae formation (18.6%) was similar to that of medium containing both lactate and pyruvate (17.6%).

Table 3. Effects of lactate and pyruvate on the development of one-cell embryos to the morula stage in the absence and presence of glucose (Experiment 2)[a]

Medium[b] Sodium lactate	Medium[b] Pyruvate	No. of oocytes inseminated	No. cleaved at Day 2 (%)	No. of morulae at Day 5 (%)	Total no. of cells in the morulae Mean±SD	Total no. of cells in the morulae Range
Glucose-free mSOF						
-	-	104	51 (49.0)[c]	4 (3.8)[c]	19.8± 5.6	16-28
+	-	110	88 (80.0)[d]	25 (22.7)[d]	22.5± 8.7	16-53
-	+	105	75 (71.4)[d]	15 (14.3)[c,d]	21.2± 6.5	16-36
+	+	110	83 (75.5)[d]	27 (24.5)[d]	23.4± 6.6	16-39
mSOF Supplemented with 0.56 mM glucose						
-	-	123	59 (48.0)[c]	10 (8.1)[c]	24.3± 8.7	16-44
+	-	120	103 (85.8)[d]	23 (19.2)[d]	22.5± 5.0	16-34
-	+	97	58 (59.8)[c]	18 (18.6)[d]	24.0±10.4	16-58
+	+	102	77 (75.5)[d]	18 (17.6)[d]	24.6± 9.1	16-48

a Data are pooled from three replicates. Day 0 = day of in-vitro insemination.
b Abbreviated as follows: mSOF containing (+) or deleting (-) 3.3 mM Na lactate and 0.3 mM Na pyruvate.
c,d Percentages in the same column with different superscripts are different within the same glucose level (P<0.05).

Effects of Amino Acids and Vitamins on Development (Experiments 3a and 3b)

When 20 amino acids were added to mSOF, the percentage of morulae formation and the total number of cells in the morulae increased; however, supplementation with vitamins caused neither beneficial nor detrimental effects, as shown in Table 4. There was no increase in the percentage of blastocysts when amino acids or amino acids and vitamins were added to mSOF (Table 5). The mean number of cells for blastocysts cultured in mSOF with amino acids (111.5 cells) and amino acids + vitamins (115.5 cells) was larger than that for the control (no additive, 71.5 cells), as shown in Table 5.

Effect of Protein in SOF and Glucose Added Post-compaction on Development to Blastocysts (Experiment 4)

Embryonic development in mSOF containing PVA instead of BSA was markedly lower than that in mSOF containing BSA (Table 6). There was no benefit gained by adding 5.56 mM glucose on Day 5 which is in contrast to the detrimental effects found when glucose is added from the start of culture (Table 2). The percentage of blastocysts (28.1%) resulting from culture of embryos with mSOF containing BSA was not different from co-culture with bovine oviduct cells (22.0%). However, the mean number of cells in blastocysts cultured in the mSOF containing BSA (109.0 cells) was lower than that of co-culture (141.4 cells).

Table 4. Effects of amino acids and vitamins on the development of one-cell embryos to the morula stage (Experiment 3a)[a]

Medium[b] AAs	Medium[b] Vits	No. of oocytes inseminated	No. cleaved at Day 2 (%)	No. of morulae at Day 5 (%)	Total no. of cells in morulae Mean±SD	Total no. of cells in morulae Range
-	-	120	97 (80.8)	34 (28.3)[c]	25.3± 7.4[e]	16-47
+	-	110	90 (81.8)	51 (46.4)[d]	31.8±11.3[f]	16-65
-	+	122	105 (86.1)	32 (26.2)[c]	28.6±9.4[e,f]	16-56
+	+	114	96 (84.2)	43 (37.7)[c,d]	32.5±11.8[f]	16-64

a Data are pooled from three replicates. Day 0 = day of in-vitro insemination.
b Abbreviated as follows: mSOF with (+) or without (-) amino acids (AAs - 1.0 mM glutamine; BME - essential amino acids and MEM - nonessential amino acids) and MEM vitamins (Vits).
c,f Values in the same column with different superscripts are different (P<0.05).

Table 5. Effects of amino acids and vitamins on the development of one-cell embryos to the blastocyst stage (Experiment 3b)[a]

Medium[b] AAs	Medium[b] Vits	No. of oocytes inseminated	No. cleaved at Day 2 (%)	No. of blastocysts at Day 7 (%)	Total no. of cells in blastocysts Mean±SD	Total no. of cells in blastocysts Range
-	-	156	140 (89.7)	32 (20.5)	71.5±21.7[c]	31-107
+	-	169	142 (84.0)	46 (27.2)	111.5±43.3[d]	46-194
+	+	169	142 (84.0)	44 (26.0)	115.5±35.2[d]	30-180

a Data are pooled from four replicates. Day 0 = day of in-vitro insemination.
b mSOF abbreviated the same as in Table 4.
c,d Values with different superscripts are different (P<0.01).

DISCUSSION

The following are conclusions that were drawn from the present experiments. Glucose is not only unnecessary but at 5.56 mM, but it causes detrimental effects on early bovine embryonic development (Tables 2 and 6). However, Robl et al. (19), showed glucose improved development of bovine embryos after the eight-cell stage. Lactate (3.3 mM) is capable of supporting development of one-cell bovine embryos, as is pyruvate (0.3 mM) in the absence of glucose. Lactate also plays an important role in first cleavage in the presence of 0.56 mM glucose (Table 3). This is similar to the findings of Rosenkrans et al. (10), who reported that lactate was the preferred substrate for the development of early bovine embryos. Amino acids (a mixture of 1.0 mM glutamine, BME essential amino acids, and MEM nonessential amino acids) enhanced early embryonic development, although the vitamins of MEM had neither beneficial nor harmful effects (Tables 4 and 5); both these findings are similar to the results of Rosenkrans and

First (20). Bovine serum albumin supported in-vitro development, and it could not be replaced with PVA under the culture conditions of our present study (Table 6).

Table 6. Effects of BSA and medium supplementation with glucose from Days 5 to 7 on the development of one-cell embryos to the blastocyst stage (Experiment 4)[a]

Medium[b]		No. of oocytes inseminated	No. cleaved at Day 2 (%)	No. of blastocysts at Day 7 (%)	Total no. of cells in blastocysts	
Mmol	Glc				Mean±SD	Range
mSOF + AAs						
BSA	-	115	89 (77.4)	33 (28.7)[c]	109.0±40.0[d]	34-171
BSA	+	110	85 (77.3)	20 (18.2)[c]	102.5±33.8	66-170
PVA	-	107	85 (79.4)	9 (8.4)[d]	75.3±39.1	32-164
PVA	+	86	65 (75.6)	3 (3.5)[d]	93.0±46.0	63-146
Co-culture with oviductal tissue in TCM199 + 10% FCS						
		227	182 (80.2)	50 (22.0)[c]	141.4±49.2[d]	42-252

a Data are pooled from three to four replicates. Day 0 = day of in-vitro insemination.
b In-vitro inseminated oocytes were cultured for 4 days in a mSOF + AAs (see Table 4) containing 3 mg/ml BSA or substituting 1 mg/ml PVA as a macromolecule (Mmol), then they were transferred to a fresh medium with (+) or without (-) 5.56 mM glucose (Glc) at Day 5. One-cell embryos were also co-cultured with oviductal tissue in TCM199 + 10% FCS for 6 days.
c,d Values in the same column with different superscripts are different ($P<0.01$).

Glycolytic activity in early mouse embryos may be suppressed because of the absence of phosphofructokinase activity (21,22). Consequently, glucose does not support development of mouse embryos until the eight-cell stage (23,24). In ruminants, the inability of glucose to promote development of eight-cell sheep embryos or 16-cell and morula bovine embryos was reported by Boone et al. (25). Our present experiments clearly demonstrate that glucose, at concentrations of 0.56 to 5.56 mM, has no beneficial effect on the development of one-cell bovine embryos (Table 2). There is no necessity for glucose in culture medium designed to promote the development of one-cell bovine embryos to the morulae stage (Table 2). Present results are supported by recent biochemical studies described by Rieger and Guay (26) who studied metabolism in Day 7 bovine blastocysts and suggested a blocking of glycolytic activity. Another study by Javed and Wright (27) has shown that total glucose utilization in bovine embryos was low from the 6- to 16-cell stages, and increased drastically at the morula stage. Results from the above two reports do not completely agree with each other, but glucose utilization by the Embden-Meyerhof pathway seems to be very low up to the morula stage.

Glucose in bovine oviductal fluid is reported to be 0.05 to 0.2 mM (28,29). Early bovine embryos can develop to the morula and blastocyst stages in the oviducts of rabbits (30) or sheep (31), where the concentration of glucose is around 1.5 mM (32,33). Glucose is usually employed for embryo culture media at a concentration of 5.56 mM based on the concentration of glucose in FCS. This level is similar to that in mouse oviduct fluid (5.2 mM; 34). In the present experiment, the presence of 5.56 mM glucose clearly inhibited embryonic development to the morula stage (Table 2). This finding of an inhibitory effect of glucose is similar to recent studies in hamster (35-37) and mouse embryos (38) but differs from results of similar studies with rat (39) and pig embryos (40). Although the inhibitory effect of glucose in cattle and the different responses to glucose among other species are not clear, glucose has been proposed to induce respiratory inhibition in the presence of phosphate (i.e., the "Crabtree effect"; 35,41). This effect may result in inefficient ATP production (35) and would inhibit lactate/amino acid-mediated development, as has been suggested for hamster embryos (36,37). Hence, it is strongly suggested that the high concentration of glucose (5.56 mM) in embryo culture media has been one of the main factors causing the 8- to 16-cell block in bovine embryos cultured in vitro.

A drastic increase in glucose utilization of bovine embryos at the morula stage has been recently reported by Javed and Wright (27). Robl et al. (19) also showed that glucose addition to a co-culture medium at 72 hours following in-vitro insemination resulted in higher developmental rate to the blastocyst stage. In our work, however, addition of 5.56 mM glucose on Day 5 showed neither a beneficial nor detrimental effect on embryonic development in vitro (Table 6). It is possible that glucose at a higher level, such as 5.56 mM, might have a latent harmful effect, even at the morula stage, and that glucose at a lower level might be beneficial for the embryonic development in vitro. Further experiments are needed to elucidate the influence of glucose on the development of bovine embryos, especially at the morula and blastocyst stages, by adding various concentrations of glucose at various times.

The concentration of lactate in sheep and rabbit oviductal fluids was reported to be 2 to 4 mM (32,33,42), which is lower than in media commonly used for culture of mammalian embryos (20 to 30 mM). In the present experiment, a lactate level of 3.3 mM (9,42) was employed, since in a preliminary experiment (unpublished data), a higher concentration of lactate (33.0 mM), similar to the level in CZB medium (31.3 mM) (38), clearly inhibited bovine embryonic development. Even when one-cell bovine embryos were cultured with high lactate in a glucose-free medium, only 5.9% of oocytes fertilized in vitro (n=51) developed beyond the 16-cell stage.

Early studies with mouse embryos have demonstrated that during the first cell cycle, mouse embryos can utilize pyruvate, but not lactate, in the absence of glucose, due to a higher ATP/ADP ratio (43,44). In contrast, to the studies on mouse embryos, we find that 3.3 mM lactate as well as 0.3 mM pyruvate (Table 3) can support one-cell bovine embryos. These results are similar to those of Rosenkrans et al. (10). A high ATP/ADP ratio inhibits the movement of reducing equivalents through the electron transport chain, thus limiting the availability of NAD+ that is necessary for the conversion of lactate to pyruvate by lactate dehydrogenase (45). Hence, the present results suggest that the ATP/ADP ratio in the one-cell bovine embryos in our study may be lower so that they can oxidize lactate to pyruvate using NAD+.

In our Experiment 2b, it is shown that deletion of lactate from a medium containing 0.56 mM glucose led to a reduction in the frequency of embryos completing the first cleavage (Table 3). This finding is similar with results on mouse embryos reported by

Spindle (46), in which removal of lactate, during the first cell cycle, from culture medium containing glucose and pyruvate reduced the development of mouse embryos. A proper lactate/pyruvate ratio has been indicated to be essential for balancing the oxidation-reduction potential of embryos (24,44). From these results, it is suggested that the presence of glucose at a lower level (0.56 mM) may not be harmful to embryonic development by itself but would change the oxidation-reduction potential so that pyruvate becomes incapable of supporting the first cell cycle. Our experiments were not factorially arranged to study interactions of all media components. It may be that the damaging effects of excess glucose are due to imbalances in ionic media constituents that influence metabolism, as has been shown for the mouse by Lawitts and Biggers (47).

One of the most salient findings from this study is that supplementation with 20 amino acids dramatically improved the development of one-cell embryos to the morula stage (Table 5). It has been reported that 5 to 6 days after estrus, bovine embryos normally develop to the morula stage (48). Embryos recovered 5 days after ovulation at the morula stage of poor, fair and good quality contained 23, 27 and 35 cells, respectively (49). In the present study, the mean number of cells in morulae which were cultured in mSOF supplemented with amino acids (31.8 cells) or with both amino acids and vitamins (32.5 cells) was comparable.

Amino acids have been reported to be essential for in-vitro development of early embryos in rabbits (50), hamsters (15), rats (39) and pigs (51). In cattle, there have been no comparable reports about the effects of exogenous amino acids on early embryonic development in vitro; however, a total of 20- and 25-free amino acids were found in oviductal fluid (52) and in uterine fluid (53), respectively. It is not clear how amino acids improve in-vitro embryonic development, but some amino acids such as glutamine may act as an energy source and indeed Rieger and Guay (26) have shown that glutamine can be metabolized by bovine blastocysts. Supplementation of amino acids to a culture medium may also increase the pool size of endogenous amino acids and de novo protein synthesis (39). Transcription of the bovine embryonic genome begins at the four-cell stage (54). The rates of protein and RNA synthesis increase dramatically between the 16-cell and the blastocyst stages in cattle, and incorporation of methionine also increases progressively from the 16-cell stage (55).

The addition of 20 amino acids also caused an increase in the number of cells of blastocysts (71.5 cells for the control and 111.5 cells for amino acid supplementation; Table 5). McLaughlin et al. (13) reported that in-vivo fertilized one-cell embryos developed to the blastocyst stage after in-vitro culture for 6 days in SOF supplemented with human serum, and that the blastocysts contained approximately 70 cells. This value is the same as that of blastocysts cultured for 6 days in the presence of mSOF without supplementation of amino acids. We have not determined the viability of the present blastocysts by transferring them to recipient cattle, but their total cell numbers 7 days after in-vitro insemination, especially those cultured in mSOF supplemented with amino acids, were similar or higher than those for recent studies in which in-vitro fertilized embryos were co-cultured with oviductal or cumulus cells. The number of cells in the blastocysts were reported to be 70 to 150 cells at 8 to 10 days after in-vitro insemination (56-58).

Bovine blastocysts are usually recovered at 7 to 8 days after estrus, and their cell numbers at the early, expanding and fully expanded blastocyst stages are reported to be approximately 100, 120 and 160 cells, respectively (48,59). The blastocysts obtained from the present co-culture system had approximately 140 cells 7 days after in-vitro insemination. This value is similar to that for in vivo-developed blastocysts. There was an increase in the cell number with the addition of amino acids to mSOF, as described

above, although this value was still smaller than that for in vivo-developed blastocysts and the co-cultured embryos in the present study.

Vitamins play vital roles as coenzymes in carbohydrate or amino acid metabolism (60) and can be expected to be required by any mammalian cell. A certain group of vitamins was reported to be necessary for blastocyst expansion and/or hatching in rabbit (50,61,62) and hamster embryos (63,64) in the presence of amino acids. In our present work, the addition of vitamins of MEM did not enhance the development of one-cell bovine embryos to the morula stage (Table 4). This result is similar to previous work in mouse (38,65), hamster (66) and rabbit embryos (50,61). Further experiments are needed to elucidate the influences on embryo development of individual vitamins and amino acids and their proper dosages, as demonstrated for hamster embryos (63,64,66).

There is a macromolecular requirement for embryo culture usually filled by the addition of BSA. It has been reported that BSA can be replaced with polyvinylpyrollidone or PVA for culture of early hamster (63), mouse (65), rabbit (62) and rat embryos (39). The present trial to culture one-cell bovine embryos in a chemically defined medium with PVA replacing BSA resulted in poor development to the blastocyst stage (8.4%; Table 6). Bovine serum albumin is not incorporated into cells of mouse embryos until the expanded blastocyst stage (67), and it has been thought to have beneficial effects on embryonic development with its great ligand-binding abilities. These benefits include protective effects against toxic components of the medium and growth promoting effects with its components (68). It is suggested that mSOF, even with amino acids, might include component(s) inhibitory for embryonic development. Alternatively, the present crystallized and lyophilized BSA may contain an unknown growth-promoting factor(s) (68).

In conclusion, our present study clearly demonstrated that one-cell bovine embryos can be cultured successfully beyond the 8- to 16-cell block to the blastocyst stage, using a semi-chemically defined medium without glucose but containing pyruvate, lactate, amino acids, and BSA. Frequency of embryonic development up to the morulae stage in this medium is at least equivalent to that for co-culture with oviductal cells, although the mean number of cells in blastocysts might be lower. A direct comparison was not performed; however, the mean number of cells in blastocysts for the present medium was lower than that for co-cultured blastocysts. Embryo culture in a chemically-defined medium in which PVA was substituted for BSA resulted in poor development, suggesting the presence of an embryotoxic component or a lack of nutrients and/or growth factor(s), such as insulin, fibroblast growth factor, or transforming growth factor-β which might stimulate and regulate mitosis of embryonic bovine cells (69-70).

REFERENCES

1. Wright, R.W., Jr. and Bondioli, K.R. Aspects of in vitro fertilization and embryo culture in domestic animals, J. Anim. Sci. 53:702-729 (1981).

2. Thibault, C. La culture in vitro de l'oeuf de vache. Ann. Biol. Anim. Biochim. Biophys. 6:159-164 (1966).

3. Camons, S., Heyman, Y., Meziou, W. and Menezo, Y. Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. J. Reprod. Fertil. 72: 479-485 (1984).

4. Eyestone, W.H. and First, N.L. A study of the 8- to 16-cell developmental block in bovine embryos cultured in vitro. Theriogenology 25:152 abstr. (1986).

5. Eyestone, W.H., Vignieri, J. and First, N.L. Co-culture of early bovine embryos with oviductal epithelium. Theriogenology 27:228 abstr. (1987).

6. Kajihara, Y., Goto, K., Nakanishi, Y. and Ogawa, K. In vitro fertilization of bovine follicular oocytes and their development up to hatched blastocysts in vitro. Jpn. J. Anim. Reprod. 33:173-180 (1987).

7. Eyestone, W.H. and First, N.L. Co-culture of early bovine embryos with oviductal tissue or in conditioned medium. J. Reprod. Fertil. 85:715-720 (1989).

8. Northey, D.L., Leibfried-Rutledge, M.L., Nuttleman, P.R. and First, N.L. Development of bovine nuclear transfer embryos in vivo vs in vitro. Theriogenology 37:266 abstr. (1992).

9. Tervit, H.R., Whittingham, D.G. and Rowson, L.E.A. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30:493-497 (1972).

10. Rosenkrans, C.F., Jr. Zeng, G.Z., Schoff, P.K. and First, N.L. A simple medium for in vitro development of bovine embryos. J. Anim. Sci. 68(Suppl):430 abstr. (1990).

11. Walker, S.K., Seamark, R.F., Quinn, P., Warnes, G.M., Ashman, R.J., Smith, D.K. and Ancell, P. Culture of pronuclear embryos of sheep in a simple medium. Proc. 11th Int. Cong. Anim. Reprod. AI, 4:483 abstr. (1988).

12. Walker, S.K., Lampe, R.J. and Seamark, R.F. Culture of sheep zygotes in synthetic oviduct fluid medium with different concentrations of sodium bicarbonate and HEPES. Theriogenology 32:797-804 (1989).

13. McLaughlin, K.J., McLean, D.M., Stevens, G., Ashman, R.A., Lewis, P.A., Bartsch, B.D. and Seamark, R.F. Viability of one-cell bovine embryos cultured in synthetic oviduct fluid medium. Theriogenology 33:1191-1199 (1990).

14. Thompson, J.G.E., Simpson, A.C., Pugh, P.A., Donnelly, P.E. and Tervit, H.R. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. J. Reprod. Fertil. 89:573-578 (1990).

15. Bavister, B.D., Leibfried, M.L. and Lieberman, G. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol. Reprod. 28:235-247 (1983).

16. Leibfried, M.L. and First, N.L. Characterization of bovine follicular oocytes and their ability to mature in vitro. J. Anim. Sci. 48:76-86 (1979).

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

18. Brackett, B.G. and Oliphant, G. Capacitation of rabbit spermatozoa in vitro. Biol. Reprod. 12:260-274 (1975).

19. Robl, J.M., Dobrinsky, R.J. and Duby, R.T. The effect of protein supplements, phosphate and glucose on the in vitro development of IVM-IVF bovine oocytes. Theriogenology 35:263 abstr. (1991).

20. Rosenkrans, C.F., Jr. and First, N.L. Culture of bovine zygotes to the blastocyst stage: effects of amino acids and vitamins. Theriogenology 35:266 abstr. (1991).

21. Barbehenn, E.K., Wales, R.G. and Lowry, O.H. The explanation for the blockage of glycolysis in early mouse embryos. Proc. Nat. Acad. Sci. (USA) 71:1056-1060 (1974).

22. Barbehenn, E.K., Wales, R.G. and Lowry, O.H. Measurement of metabolites in single preimplantation embryos; a new means to study metabolic control in early embryos. J. Embryol. Exp. Morph. 43:29-46 (1978).

23. Whitten, W.K. Culture of tubal ova. Nature 179:1081-1082 (1957).

24. Brinster, R.L. Studies on the development of mouse embryos in vitro. II. The effects of energy source. J. Exp. Zool. 158:59-68 (1965).

25. Boone, W.R., Dickey, J.F., Luszcz, L.J., Dantzler, J.R. and Hill, J.R., Jr. Culture of ovine and bovine ova. J. Anim. Sci. 47:908-913 (1978).

26. Rieger, D. and Guay, P. Measurement of the metabolism of energy substrates in individual bovine blastocysts. J. Reprod. Fertil. 83:585-591 (1988).

27. Javed, M.H. and Wright, R.W.J. Determination of pentose phosphate and Embden-Meyerhof pathway activities in bovine embryos. Theriogenology 35:1029-1037 (1991).

28. Carlson, D., Black, D.L. and Howe, G.R. Oviduct secretion in the cow. J. Reprod. Fertil. 22:549-552 (1970).

29. Parrish, J.J., Susko-Parrish, J.L. and First, N.L. Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. Biol. Reprod. 41:683-699 (1989).

30. Boland, M. Use of the rabbit oviduct as a screening tool for the viability of mammalian eggs. Theriogenology 21:126-137 (1984).

31. Eyestone, W.H., Leibfried-Rutledge, M.L., Northey, D.L., Gilligan, B.L. and First, N.L. Culture of one-cell and two-cell bovine embryos to the blastocyst stage in the ovine oviduct. Theriogenology 28:1-7 (1987).

32. Iritani, A., Gomes, W.R. and Vandemark, N.L. Secretion rates and chemical composition of oviduct and uterine fluids in ewes. Biol. Reprod. 1:72-76 (1961).

33. Leese, H.J. The formation and function of oviduct fluid. J. Reprod. Fertil. 82:843-856 (1988).

34. Gardner, D.K. and Leese, H.J. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism. J. Reprod. Fertil. 88:361-368 (1990).

35. Schini, S.A. and Bavister, B.D. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol. Reprod. 39:1183-1192 (1988).

36. Seshagiri, P.B. and Bavister, B.D. Phosphate is required for inhibition by glucose of development of hamster 8-cell embryos in vitro. Biol. Reprod. 40:607-614 (1989).

37. Seshagiri, P.B. and Bavister, B.D. Glucose inhibits development of hamster 8-cell embryos in vitro. Biol. Reprod. 40:599-606 (1989).

38. Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L. and Torres, I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J. Reprod. Fertil. 86:679-688 (1989).

39. Zhang, X. and Armstrong, D.T. Presence of amino acids and insulin in a chemically defined medium improved development of 8-cell rat embryos in vitro and subsequent implantation in vivo. Biol. Reprod. 42:662-668 (1990).

40. Petters, R.M., Johnson, B.H., Reed, M.L. and Archibong, A.E. Glucose, glutamine and inorganic phosphate in early development of the pig embryo in vitro. J. Reprod. Fertil. 89:269-275 (1990).

41. Crabtree, H.G. Observation on the carbohydrate metabolism of tumours. Biochem. J. 29:536-545 (1929).

42. Restall, B.J. and Wales, R.G. The fallopian tube of the sheep. III. The chemical composition of the fluid from the fallopian tube. Aust. J. Biol. Sci. 19:687-698 (1966).

43. Biggers, J.D., Whittingham, D.G. and Donahue, R.P. The pattern of energy metabolism in the mouse oocyte and zygote. Proc. Nat. Acad. Sci. (USA) 58:560-567 (1965).

44. Cross, P.C. and Brinster, R.L. The sensitivity of one-cell mouse embryos to pyruvate and lactate. Exp. Cell Res. 77:57-62 (1973).

45. Quinn, P. and Wales, R.G. Uptake and metabolism of pyruvate and lactate during preimplantation development of the mouse embryo in vitro. J. Reprod. Fertil. 35:273-287 (1973).

46. Spindle, A. In vitro development of one-cell embryos from outbred mice: influence of culture medium composition. In Vitro Cell Dev. Biol. 25:151-156 (1990).

47. Lawitts, J.A. and Biggers, J.D. Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biol. Reprod. 45:245-251 (1991).

48. Betteridge, K.J. and Flechon, J.E. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 29:155-187 (1988).

49. Wurth, Y.A., van der Zee-Kotting, W., Kruip, Th.A.M., Dieleman, S.J. and Bevers, M.M. Relation between microscopic quality of bovine embryos and number of blastomeres. Proc. 11th Int. Cong. Anim. Reprod. A.I. 3:352 abstr. (1988).

50. Kane, M.T. and Foote, R.H. Culture of two- and four-cell rabbit embryos to the expanding blastocyst stage in synthetic media. Proc. Soc. Exp. Biol. Med. 133:921-925 (1970).

51. Rosenkrans, C.F., Jr., Davis, D.L. and Milliken, G. Pig blastocyst development in vitro is affected by amino acids. J. Anim. Sci. 67:1503-1508 (1989).

52. Stanke, D.F., Sikes, J.D., DeYoung, D.W. and Tumbleson, M.E. Proteins and amino acids in bovine oviductal fluid. J. Reprod. Fertil. 38:493-496 (1974).

53. Fahning, M.L., Schultz, R.H. and Graham, E.F. The free amino acid content of uterine fluids and blood serum in the cow. J. Reprod. Fertil. 13:229-236 (1967).

54. Barnes, F.L. and Eyestone, W.H. Early cleavage and the maternal zygotic transition in bovine embryos. Theriogenology 33:141-152 (1990).

55. Frie, R.E., Schultz, G.A. and Church, R.B. Qualitative and quantitative changes in protein synthesis occur at the 8 to 16-cell stage of embryogenesis in the cow. J. Reprod. Fertil. 86:637-641 (1989).

56. Kajihara, Y., Goto, K., Tokumaru, M., Koba, M., Nakanishi, Y. and Ogawa, K. Number of blastomeres and chromosome abnormalities of bovine blastocysts obtained from in vitro matured, fertilized, and cultured follicular oocytes. Jpn. J. Anim. Reprod. 34:191-198 (1988).

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

58. Iwasaki, S. and Nakahara, T. Cell number and incidence of chromosomal anomalies in bovine blastocysts fertilized in vitro followed by culture in vitro or in vivo in rabbit oviducts. Theriogenology 33:669-675 (1990).

59. Mannaerts, B.M.J.L. Cytological parameters for rating bovine embryo quality. In: Sreenan, J.M. and Diskin, M.G. (eds), Embryonic Mortality in Farm Animals. Martinus Nijhoff Publisher, Dordrecht, 1986, pp. 216-222.

60. Lehninger, A.L. Biochemistry. Worth Publishers, New York, 1975.

61. Kane, M.T. Culture of mammalian ova. In: Sreenan, J.M. (ed), Control of Reproduction in the Cow. Martinus Nijhoff, The Hague, 1978, pp. 383-397.

62. Kane, M.T. The effects of water-soluble vitamins on the expansion of rabbit blastocysts in vitro. J. Exp. Zool. 245:220-223 (1988).

63. Kane, M.T. and Bavister, B.D. Protein-free culture medium containing polyvinylalcohol, vitamins, and amino acids supports development of eight-cell hamster embryos to hatching blastocysts. J. Exp. Zool. 247:183-187 (1988).

64. Kane, M.T., Carney, E.W. and Bavister, B.D. Vitamins and amino acids stimulate hamster blastocysts to hatch in vitro. J. Exp. Zool. 239:429-432 (1986).

65. Cholew, J.A. and Whitten, W.K. Development of two-cell mouse embryos in the absence of fixed-nitrogen source. J. Reprod. Fertil. 22:553-555 (1970).

66. Bavister, B.D. and Arlotto, T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. Mol. Reprod. Dev. 25:45-51 (1990).

67. Smith, A.L. and Carson, S.A. Incorporation of fluoroscein-isothiocyanate-labelled bovine serum albumin into murine embryos during in vitro culture. Theriogenology 31:259 abstr. (1989).

68. Kane, M.T. In vitro growth of preimplantation rabbit embryos. In: Bavister, B.D. (ed), The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation In Vitro. Plenum Press, New York, 1987, pp. 193-217.

69. Marquant Le Guienne, B., Gerard, M., Solari, A. and Thibault, C. In vitro culture of bovine eggs fertilized either in vivo and in vitro. Reprod. Nutr. Dev. 29:559-568 (1989).

70. Larson, R.C., Ignotz, G.G. and Currie, W.B. Defined medium containing TGF, and bFGF permits development of bovine embryos beyond the "8-cell block." J. Reprod. Fertil. Series 5:22 abstr. (1990).

Theriogenology 37:979-991, 1992

SURVIVAL OF EQUINE EMBRYOS CO-CULTUREO WITH EQUINE OVIDUCTAL EPITHELIUM FROM THE FOUR- TO EIGHT-CELL TO THE BLASTOCYST STAGE AFTER TRANSFER TO SYNCHRONOUS RECIPIENT MARES[1]

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ABSTRACT

In this study we examined the ability of equine oviductal epithelial cells (OEC) to support the development of four- to eight-cell equine embryos in vitro and investigated the ability of co-cultured embryos to continue normal development after transfer to synchronous recipient mares. Equine embryos obtained at Day 2 after ovulation were cultured with or without OEC for 5 days. Those OEC co-cultured embryos that reached the blastocyst stage and embryos recovered from the uterus at Day 7 were surgically transferred to synchronous recipient mares.

Co-culture with OEC improved ($P < 0.01$) development of four- to eight-cell embryos to blastocysts compared to medium alone (11/15 vs 0/6) during 5 days in vitro. Embryos co-cultured with OEC were smaller ($P < 0.05$) and more delayed in development than Day-7 uterine blastocysts. There was no difference in the Day-30 survival rate of co-cultured blastocysts (3/8) or Day-7 uterine blastocysts (5/8) after transfer to recipient mares. These results indicate that co-culture with OEC can support development of four- to eight-cell equine embryos in vitro and that co-cultured embryos can continue normal development after transfer to recipient mares.

Key words: equine, embryo culture, oviduct, co-culture, embryo transfer

INTRODUCTION

Early cleavage stage embryos from a number of mammalian species undergo developmental blocks in vitro (1). Co-culture with oviductal tissue enhances the in vitro development of early cleavage stage embryos in domestic species, including cattle, sheep, pigs and horses (2-10). Although the mechanism of action of oviductal

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Oocyte and Embryo Quality: Effect of Origin, Culture Conditions and Gene Expression Patterns

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Contents

In general, the majority of immature bovine oocytes fail to develop to the blastocyst stage following maturation, fertilization and culture *in vitro*. The evidence suggests that while culture conditions during *in vitro* embryo production can impact on the developmental potential of the early embryo, the intrinsic quality of the oocyte is the key factor determining the proportion of oocytes developing to the blastocyst stage. In addition, evidence suggests that the period of post-fertilization embryo culture is the most critical in determining blastocyst quality. This paper reviews the current literature, with emphasis on the bovine model, demonstrating evidence for an effect of oocyte origin and/or *in vitro* maturation conditions on the developmental capacity and gene expression patterns in the oocyte. Furthermore, the well-documented effects of post-fertilization culture environment on embryo gene expression and quality are highlighted.

Introduction

The development of immature bovine oocytes to the blastocyst stage following maturation, fertilization and culture *in vitro* is generally limited to a frequency of about 40%. There is now a growing amount of evidence to suggest that while culture conditions during *in vitro* embryo production can impact on the developmental potential of the early embryo, the intrinsic quality of the oocyte is the key factor determining the proportion of oocytes developing to the blastocyst stage. Recent studies from our laboratory (Rizos et al. 2002b; Rizos et al. 2002c; Rizos et al. 2003), comparing bovine oocyte maturation, fertilization and embryo culture *in vivo* and *in vitro* have demonstrated that the origin of the oocyte is the main factor affecting blastocyst yield while the post-fertilization culture environment is critical in determining blastocyst quality, measured in terms of cryotolerance (Rizos et al. 2002c; Rizos et al. 2003) and relative transcript abundance (Rizos et al. 2002b, 2003), irrespective of the origin of the oocyte.

It is generally accepted that bovine embryos produced *in vitro* have a lower developmental capacity following transfer than do embryos produced *in vivo*. Large field trials under commercial conditions have shown that pregnancy rates after transfer of both frozen–thawed and fresh *in vitro*-produced embryos are generally significantly reduced (Hasler et al. 1995; Galli and Lazzari 1996; van Wagtendonk-de Leeuw et al. 2000).

Production of bovine embryos *in vitro* is essentially a three-step process involving oocyte maturation, oocyte fertilization and subsequent culture of the *in vitro*-derived zygote. Deviations compared with *in vivo*-produced embryos may originate from any of these steps. In terms of efficiency, approximately 90% of immature oocytes undergo nuclear maturation *in vitro*; about 80% undergo fertilization and cleave at least once, to the two-cell stage. However, as mentioned above, the majority fails to reach the blastocyst stage. Thus, the major fall-off in development occurs during the last part of the process, between the zygote and blastocyst stages, suggesting that post-fertilization embryo culture is the most critical period of the process in terms of determining the blastocyst yield. However, as discussed below, there is now unequivocal evidence demonstrating that events further back along the developmental axis are crucial in determining the proportion of immature oocytes that form blastocysts and that in fact the post-fertilization culture environment, within certain limits, does not affect the capacity of the immature oocyte to form a blastocyst.

The aim of this paper is to review the literature, with emphasis on the bovine model, demonstrating evidence for an effect of oocyte origin and/or *in vitro* maturation conditions on the developmental capacity and gene expression patterns of the oocyte and also to highlight the well-documented effects of post-fertilization culture environment on embryo gene expression and quality. At the outset, it is important to define what is meant by the term 'quality'. The ultimate test of the quality of an oocyte is its ability to be fertilized and develop to the blastocyst stage, to establish a pregnancy and to produce a live offspring. Similarly, the best measure of blastocyst quality is its ability to establish a pregnancy and produce a live offspring. From a research standpoint, because of the impracticality of transferring every embryo, attainment of the blastocyst stage is generally used as an indicator of oocyte developmental competence. For the same reason, other methods of assessing blastocyst quality are used, including cell counts, cryotolerance and, latterly, gene expression patterns.

The Oocyte

The intrinsic quality of the oocyte determines its developmental capacity

In the cyclic cow, final maturation of the ovulatory follicle is initiated by the pre-ovulatory LH surge. During the subsequent 24-h period, the oocyte nucleus progresses from prophase I to metaphase II. In addition several changes occur at the level of the cytoplasm involving redistribution of intracellular organelles

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(Hyttel et al. 1986a; Hyttel et al. 1986b; Hyttel et al. 1989; Hyttel et al. 1997). *In vitro*, resumption of meiosis occurs spontaneously on removal from the follicle (Pincus and Enzmann 1935) and the majority of fully-grown oocytes progress to metaphase II following 24-h culture (Fair et al. 1995; Lonergan et al. 1997). However, it is widely believed that the corresponding cytoplasmic maturation is compromised, leading to the poor developmental rates.

There is much evidence to indicate that the origin of the oocyte is crucial in determining its ability to at least undergo fertilization, cleavage and development to the blastocyst stage. For example, oocytes derived from large follicles are more competent than those derived from small follicles (Pavlok et al. 1992; Lonergan et al. 1994). In addition, there are a number of reports in the literature indicating that oocytes matured *in vivo* are more competent that those matured *in vitro* (Greve et al. 1987; Leibfried-Rutledge et al. 1987; Marquant-Le Guienne et al. 1989; Van Soom et al. 1992; Bordignon et al. 1997; van de Leemput et al. 1999; Rizos et al. 2002c).

While there is some evidence that modifying conditions of *in vitro* maturation can affect the subsequent blastocyst yield, improvements are generally modest and even in the best cases, approximately half of the oocytes fail to reach that stage. Some authors have attempted to improve oocyte developmental competence by artificially maintaining oocytes in meiotic arrest *in vitro* for varying periods (Kubelka et al. 2000; Lonergan et al. 2000a; Sirard 2001). Thus far, while it is possible to reversibly inhibit meiotic resumption without having any adverse effect on blastocyst formation rate, there is little evidence for a positive effect on oocyte competence. Others have attempted to modify the follicular environment of the oocyte before recovery, in an effort to improve developmental competence. A 'coasting' period between hormonal stimulation and ovary collection (Blondin et al. 1997b) as well as the time interval between ovary collection and oocyte aspiration (Blondin et al. 1997a) have been shown to significantly affect the developmental competence of the oocyte. In addition, donors were administered six doses of FSH and an injection of LH, before ovum pick up approximately 80% of the recovered oocytes formed blastocysts following *in vitro* procedures (Blondin et al. 2002). Such high developmental rates are certainly encouraging and highlight the importance of oocyte quality at the start of the *in vitro* embryo production process in determining the final outcome.

In a number of studies comparing *in vivo* and *in vitro* maturation, *in vivo*-matured oocytes were compared with those recovered from small follicles on the ovaries of slaughtered animals. The developmental histories and potential fates of such oocytes would be very different rendering the validity of their comparison questionable. For this reason, several groups, including our own have compared the developmental capacity of such oocytes having an identical pre-ovulatory history (Hendriksen et al. 2000; Dieleman et al. 2002). Rizos et al. (2002c) compared the development of oocytes recovered at the estimated time of the pre-ovulatory surge and matured *in vitro* with that of their counterparts recovered 20 h later after *in vivo* maturation. Despite the fact that both groups were fertilized and cultured at the same time a significantly higher proportion of *in vivo* matured oocytes formed blastocysts 58.2% vs 39.2%). Similarly, in the study of van de Leemput et al. (de Leemput et al. 1999) *in vitro* development to the blastocyst stage of *in vivo*-matured oocytes was twice as high as that of *in vitro*-matured oocytes.

Does the site of fertilization (*in vivo* vs *in vitro*) affect developmental competence?

Further evidence for the fact that the intrinsic quality of the oocyte determines the blastocyst yield comes from the results of experiments examining the developmental rates of *in vivo*-produced zygotes or early embryos collected *in vivo* and subsequently cultured *in vitro*. In one such study from our group *in vitro* matured/fertilized (IVM/IVF) oocytes derived from 2–8 mm follicles on slaughterhouse ovaries resulted in a blastocyst yield of approximately 35% irrespective of whether the zygotes were cultured *in vitro* or *in vivo* in the sheep oviduct (Rizos et al. 2002c). In the reciprocal experiment, >70% of *in vivo* matured/fertilized oocytes reached the blastocyst stage following culture either *in vitro* or *in vivo*. Similarly, in the study by McCaffrey et al. (1991), approximately 60–80% of *in vivo*-derived 1–4-cell bovine embryos developed to morulae/blastocysts following culture *in vitro*, confirming their high developmental competence. In sheep, IVM/IVF-derived zygotes were compromised compared with *in vivo*-matured and fertilized zygotes in terms of lambing rates (25–35% vs 51–60%, respectively) (Holm et al. 1996).

It should be noted, as pointed out by Holm et al. (2002), that the behaviour of *in vivo*-derived zygotes *in vitro* is a reflection of their origin rather than an indication of how they would develop *in vivo*. These authors used time-lapse video analysis to compare the influence of *in vivo* vs *in vitro* maturation and fertilization on the *in vitro* developmental kinetics of the resulting zygotes. Zygotes capable of developing to the blastocyst stage developed faster during the first three cell cycles (by about 1–2 h). *In vivo*-derived zygotes were more developmentally competent *in vitro* than were all groups of in vitro derived zygotes; 87% vs <54% of the respective zygotes developed to the compact morula or blastocyst stages. In addition, the duration of the first cell cycles before embryonic genome activation were shorter in *in vivo*-derived than in *in vitro*-derived zygotes.

Whether this increased developmental competence is caused solely by superior maturation or is a combination of maturation and fertilization *in vivo* is not clear. Certainly, as pointed out above, there is substantial evidence in the literature to indicate that *in vivo*-matured oocytes are more competent than their *in vitro*-matured counterparts. However, evidence from our own group would support the hypothesis that the site of fertilization (*in vivo* vs *in vitro*) is also important (Rizos et al. 2002c). In that study, a higher proportion of *in vivo*-matured oocytes developed to blastocysts following fertilization *in vivo* compared with fertilization *in vitro* (73.9% vs 58.2%, respectively), despite the fact that both groups were cultured *in vitro* in parallel. However,

it should be noted that the *in vivo* fertilized oocytes were ovulated oocytes; this is in contrast with the *in vivo* matured/*in vitro* fertilized group, in which oocytes were recovered from pre-ovulatory follicles just prior to the expected time of ovulation. Whether the additional time in the follicle contributed to the improved developmetal rates is unclear. To address this question we attempted the fertilization of *in vitro* matured bovine oocytes in the sheep oviduct using gamete intrafallopian tube transfer (GIFT), involving the transfer of matured oocytes and sperm to the oviduct simultaneously, or the transfer of matured oocytes to the oviduct of a ewe previously inseminated with bovine sperm. Irrespective of the method used, only a very low proportion of oocytes were fertilized and none developed to blastocysts. Other authors have similarly attempted the *in vivo* fertilization of *in vitro*-matured bovine oocytes in the inseminated rabbit (Sreenan 1970; Hunter et al. 1972; Trounson et al. 1977), sheep (Sreenan 1970) or cow oviduct (Trounson et al. 1977; Newcomb et al. 1978; Myers et al. 1992) with limited success, although Newcomb et al. (Newcomb et al. 1978) did report the birth of twin calves following one such attempt. It would seem that such an approach is fraught with technical difficulties which only cloud the issue. One way to approach this question in a more satisfactory manner may be to use endoscopy (Besenfelder and Brem 1998; Besenfelder et al. 2001).

Importance of the kinetics of early cleavage divisions

The developmental kinetics of early bovine embryos has been studied by several authors (Grisart et al. 1994; Langendonckt et al. 1997; Holm and Callesen 1998; Holm et al. 1998; Lockwood et al. 1998; Lonergan et al. 1999; Lonergan et al. 2000b; Holm et al. 2002). We have previously demonstrated a clear relationship between the time of first cleavage post-insemination *in vitro* and developmental competence, with those oocytes cleaving earliest after *in vitro* fertilization being more likely to reach the blastocyst stage than their later-cleaving counterparts (Dinnyes et al. 1999; Lonergan et al. 1999). This phenomenon is common to many species (Bavister et al. 1983; McKiernan and Bavister 1994; Totey et al. 1996; Sakkas et al. 1998; Shoukir et al. 1998; Warner et al. 1998; Fenwick et al. 2002). In addition, we have demonstrated that this timing of first cleavage is related to the polyadenylation status of several developmentally important gene transcripts (Brevini-Gandolfi et al. 2002).Subsequently, we demonstrated differences in gene expression in the early embryo that are reflective of differences in developmental competence between early- and late-cleaving zygotes (Lonergan et al. 2000b; Fair et al. 2002). The factors that control the time of first cleavage are unclear. Although culture conditions can influence the kinetics of early development (Pinyopummintr and Bavister 1994; Langendonckt et al. 1997), it is likely that the main factors controlling this parameter are intrinsic to the oocyte (Lonergan et al. 1999; Lonergan et al. 2000b; Brevini-Gandolfi et al. 2002), the sperm (Eid and Parrish 1995; Comizzoli et al. 2000; Ward et al. 2001) or both. Indeed, in mice, a gene controlling the rate of pre-Implantation cleavage division and subsequent embryo survival (*Ped*: pre-implantation embryo development) has been identified (Warner et al. 1998).

Influence of oocyte source and/or maturation conditions on oocyte gene expression patterns

It is generally accepted that mRNA and protein molecules synthesized during oocyte growth and maturation contribute to early development prior to embryonic genome activation. The storage of mRNA takes place during oocyte growth and the extent of the poly(A) tail at the 3′ end of each transcript has emerged as an important regulatory element for determining their stability. Thus, control of polyadenylation represents a key regulatory step in gene expression and is known to be important in early embryonic development (Gandolfi and Gandolfi 2001). Conditions of *in vitro* maturation can affect the polyadenylation level in maternal mRNAs (Pocar et al. 2001).

As pointed out by De Sousa et al. (1998b), the apparent lack of developmental competence in the majority of oocytes matured *in vitro* may reflect the composition and abundance of specific transcripts in oocyte mRNA pools. Oocyte morphology associated with competence to undergo meiosis and development to the blastocyst stage can be positively correlated with a greater relative abundance of known mRNA transcripts (De Sousa et al. 1998a). However, with the exception of genes known to play roles in early ovarian folliculogenesis such as CSF-1, Connexin 37 and GDF-9, little is known of oocyte gene transcripts that may confer developmental competence to the blastocyst stage. De Sousa et al. (1998b) demonstrated a greater relative abundance of Na^+, K^+-ATPase α1 among individual bovine COCs normally selected for developmental ability compared with unselected oocytes.

While there is considerable evidence in the literature indicating that the culture environment in which the embryo develops can alter transcript levels within it (see below), there is much less corresponding information available on the influence of the follicular environment or maturation conditions *in vitro* on transcript levels in the oocyte. Such information could contribute to our understanding of the factors controlling developmental competence acquisition and potentially aid in the refinement of media for oocyte maturation *in vitro*.

While there is an increasing amount of data on gene expression patterns in oocytes at, for example, specific stages of maturation (Robert et al. 2000, 2002; Goto et al. 2002; Dalbies-Tran and Mermillod 2003; Zeng and Schultz 2003) there is a lack of information on how such patterns of expression are affected by changes in the environment of the oocyte (follicular environment or that during maturation *in vitro*). Given what we know about the dramatic effect of post-fertilization culture conditions on gene expression in the early embryo, it is not unreasonable to assume that a similar situation exists in relation to the environment during oocyte maturation. One such study by Watson et al. (2000) demonstrated that oocyte maturation media could influence levels of certain oocyte transcripts. They also

observed a relationship between transcript abundance and developmental ability to the blastocyst stage. In a recent study from our group, we contrasted the relative abundance of 12 gene transcripts in bovine oocytes matured either *in vitro* or *in vivo* (Lonergan et al. 2003); consistent with the findings of Watson et al. (2000), we observed distinct differences in the relative mRNA abundance of several developmentally important gene transcripts in bovine oocytes which are related to developmental competence. In particular, transcripts for GDF-9 were expressed at significantly higher levels in oocytes recovered at the LH surge and matured *in vitro* compared with those matured *in vivo* (Lonergan et al. 2003).

Robert et al. (2001) identified mRNA expressed in granulosa cells characterizing differentiated follicles bearing developmentally competent bovine oocytes. Dalbies-Tran and Mermillod (2003) compared gene expression patterns in oocytes before and after IVM using heterologous cDNA array hybridization: bovine cDNA probes were hybridized onto arrays of human express sequence tags (EST). About 70 transcripts underwent a significant differential regulation. Clearly, such technology could be applied to other models to characterize more fully the effect of suboptimal culture conditions.

The Embryo

Blastocyst development is only one step along the road to the production of a live offspring. As pointed out by McEvoy et al. (2000), attainment of the blastocyst stage is more a reflection of past achievement than a guarantee of future fitness. Therefore, in addition to increasing the success rate of *in vitro* embryo production (i.e. the number of oocytes developing to blastocysts) it is essential that those embryos that do reach this stage *in vitro* are of the highest quality possible in order to ensure optimal pregnancy rates following transfer. The quality of *in vitro*-produced blastocysts continually lags behind that of blastocysts produced *in vivo* and this can be linked to differences which exist between them. Compared with their *in vivo* counterparts, *in vitro* produced embryos tend to have darker cytoplasm and a lower buoyant density (Pollard and Leibo 1994) as a consequence of their higher lipid content (Abd El Razek et al. 2000), a more fragile zona pellucida (Duby et al. 1997), reduced expression of intercellular communicative devices (Boni et al. 1999), differences in metabolism (Khurana and Niemann 2000; Thompson 2000) and a higher incidence of chromosome abnormalities (Viuff et al. 1999; Slimane et al. 2000). In addition, many differences at the ultrastructural level have been reported (Crosier et al. 2000; Crosier et al. 2001; Fair et al. 2001; Crosier et al. 2002; Rizos et al. 2002a).

Post-fertilization culture environment determines blastocyst quality

By culturing *in vitro*-produced bovine zygotes *in vivo* in the ewe oviduct, it is possible to dramatically increase the quality of the resulting blastocysts, measured in terms of cryotolerance, to a level similar to that of totally *in vivo*-produced embryos (Galli and Lazzari 1996; Enright et al. 2000; Rizos et al. 2002c). Furthermore, in the reciprocal experiment, the culture *in vitro* of *in vivo*-produced bovine zygotes resulted in blastocysts of low cryotolerance (Rizos et al. 2002c). Such observations highlight the importance of the post-fertilization culture environment for the quality of the resulting blastocyst.

In support of this notion, Knijn et al. (2002) compared gene expression in blastocysts derived from *in vivo*- or *in vitro*-matured bovine oocytes. No differences were observed in the relative abundance of transcripts for four genes studied, suggesting that maturation is not the major step in the *in vitro* embryo production process affecting expression of these genes in the embryo. In other words, despite using oocytes from two sources known to differe in their developmental competence, culturing the zygotes in under the same conditions produced blastocysts of similar quality.

Gene expression

There is now a large amount of data demonstrating that the post-fertilization embryo culture environment can have a dramatic effect on the pattern of gene expression in the embryo that in turn can have serious implications for the normality of the blastocyst. This is the case, not only when one compares *in vitro* and *in vivo* culture systems, but also between different *in vitro* culture systems (Eckert and Niemann 1998; Wrenzycki et al. 1999; Doherty et al. 2000; Wrenzycki et al. 2000; Lee et al. 2001; Lequarre et al. 2001; Minami et al. 2001; Wrenzycki et al. 2001; Rief et al. 2002; Rizos et al. 2002b; Rizos et al. 2003).

In our own group, Rizos et al. (2002b) examined the expression of seven genes known to be involved in apoptosis, oxidative stress, gap junction formation and differentiation in blastocysts derived from *in vitro*-matured and -fertilized oocytes which were cultured either *in vitro* in synthetic oviduct fluid (SOF) or *in vivo* in the ewe oviduct and compared the pattern of expression with that of *in vivo*-derived blastocysts. Culture *in vitro* resulted in, amongst other differences, an elevated abundance of transcripts for Bax, a cell death-promoting factor, as well as reduced expression of the gap junction gene, Cx43. Consistent with our observations, Lazzari et al. (2002) reported that bovine embryos developed in the sheep oviduct were characterized by an expression pattern of severally developmentally important genes that was nearly identical to that of their *in vivo*-produced counterparts. Furthermore, in a comparison of serum-free and serum-supplemented SOF (Rizos et al. 2003), the presence of serum during the culture period resulted in a significant increase in the level of expression of Mn-SOD, SOX, Bax, LIF and LIF-Rβ and a decrease in the relative abundance of transcripts for Cx43 and interferon-τ.

Unlike the situation with regard to *in vitro* culture, there is very little evidence directly demonstrating an effect of alterations in the *in vivo* environment on gene expression in the pre-implantation embryo. Wrenzycki

et al. (2000) reported that the diet type fed to superovulated heifers affected the relative mRNA abundance and pyruvate metabolism of embryos; the relative abundance of Cu/Zn-SOD was affected in heifers fed barley-based diets.

Large Offspring Syndrome

As mentioned above, the detrimental effects of culture under suboptimal conditions *in vitro* are mediated through modifications of gene expression in the embryo. These detrimental effects can be seen in terms of blastocysts incapable of withstanding cryopreservation or establishing a pregnancy or, as has been shown in the case of ruminants, the production of offspring of abnormal size (Kruip and den Daas 1997; Young et al. 1998; Sinclair et al. 2000; Walker et al. 2000). This latter phenomenon is directly linked to the post-fertilization culture period *in vitro* (McEvoy et al. 2001). The existence of this syndrome demonstrates that manipulation of the environment of an embryo can alter subsequent foetal, placental and peri- and post-natal development profoundly in farm animals (McEvoy et al. 1998; Sinclair et al. 1999, 2000; van Wagtendonk-de Leeuw et al. 2000; Bertolini et al. 2002; Crosier et al. 2002).

Lazzari et al. (2002) analysed cellular (cell number on day 7, size of elongating embryo at day 12) and molecular (relative transcript abundance) parameters in bovine embryos and the incidence of large offspring syndrome in calves derived from transfer of embryos produced with different *in vitro* and *in vivo* systems. *In vitro* culture of bovine embryos in the presence of high concentrations of serum or bovine serum albumin significantly increased the number of cells in day 7 blastocysts, the size of the blastocyst on day 12 and the relative abundance of transcripts for several genes including HSP70.1, Cu/Zn-SOD, Glut-3, Glut-4, bFGF and IGFI-R when compared with embryos cultured *in vivo* (either in the sheep oviduct or produced entirely *in vivo*). These deviations were linked to gestation length and birth weight of the derived calves. Both *in vitro* systems were associated with a significantly elevated incidence of deviations in embryonic development and a higher proportion of calves with increased birthweight and other symptoms of LOS.

Chromosomal abnormalities

Viuff et al. (1999) reported that the incidence of mixoploidy was significantly higher among bovine blastocysts produced in vitro than among *in vivo* embryos from superovulated donors (78% vs 25%); furthermore 17% of *in vitro* blastocysts but none of those produced *in vivo* had a >10% incidence of polyploidy. To what extent mixoploidy is aggravated by *in vitro* culture is unknown. During development from days 2 to 5, the degree of mixoploidy increases in both *in vivo* developed and *in vitro*-derived embryos; in those derived *in vivo* however, the level of mixoploidy levels out much earlier (Viuff et al. 2000; Viuff et al. 2001).

The origin of the oocyte can affect the chromosomal constitution of the blastocyst; the degree of mixoploidy in blastocysts derived from *in vivo* matured oocytes (fertilized and cultured *in vitro*) was similar to that reported for entirely *in vivo* developed blastocysts and lower than that of oocytes recovered just prior to the pre-ovulatory LH surge and matured *in vitro*-matured (21% vs 50%)(Dieleman et al. 2002).

Temporal effects of post-fertilization culture

Several major developmental events occur during the 6-day window between zygote and blastocyst formation in cattle. These include the first cleavage division, the timing of which is known to be of critical importance in determining the subsequent development of the embryo (Lonergan et al. 1999), the activation of the embryonic genome at the 8–16-cell stage (Memli and First 2000), compaction of the morula on day 5, which involves the establishment of the first intimate cell-to-cell contacts in the embryo (Boni et al. 1999) and blastocyst formation on days 6–7, involving the differentiation of two cell types, the trophectoderm and the inner cell mass (Watson 1992). Clearly, any modifications of the culture environment, which could affect any, or all, of these processes could have a major effect on the quality of the embryo.

From the above, it is clear that the post-fertilization environment in which the embryo is cultured is critical for its normal development. There is evidence in the literature of temporal sensitivities of embryos, which would indicate, as pointed out by McEvoy et al. (2001) that some windows of embryo development are more predisposed to aberrant programming than others. The use of a two-stage culture system may avoid the conditions leading to fetal oversize. When serum was avoided during the first 3 days of sheep embryo culture *in vitro*, oversize was not observed in the resultant offspring (Kuran et al. 2000). In the same study, culture for just 2 days in SOF plus serum before transfer on day 3 to synchronous recipients also generated normal fetuses. Negrin et al. (1997) carried out an experiment involving culture for 2 days in either SOF plus serum or in co-culture, followed by culture for a further 3 days in the same or alternative system. Their observations suggested that the developmental ability of the zygotes was unaffected by the culture system used in the first 2 days, but the system used subsequently had a significant impact.

We have addressed the question of a temporal association between culture environment and embryo quality in three recent studies from our group. In the first of these (Rizos et al. 2001), the addition of granulosa cells to SOF in the form of a monolayer from day 3 onwards was necessary in order to acquire the improved cryotolerance seen in blastocysts derived from monolayer culture compared to those cultured in SOF alone. In the second study (Lonergan et al. 2002), we used a combination of *in vitro* culture in SOF and *in vivo* culture in the ewe oviduct to identify the period of post-fertilization culture that is most critical/sensitive in determining blastocyst quality. Presumptive zygotes produced by *in vitro* maturation and fertilization were cultured either *in vitro* in SOF for 6 days, *in vivo* in the oviduct for 6 days or in combinations of both. In terms

of cryotolerance, *in vivo*-produced blastocysts, those cultured *in vivo* from day 2 onwards or those cultured *in vivo* for the entire 6 days had the highest survival rates while those embryos which spent the longest period in SOF had the lowest rates of survival.

In the third study (P. Lonergan et al., in prep.) in order to try and identify at what stage the pattern of gene expression described for *in vivo*- and *in vitro*-derived blastocysts (Rizos et al. 2002b) diverges, zygotes produced by *in vitro* maturation and fertilization were divided in two groups and cultured either *in vitro* in SOF or *in vivo* in the sheep oviduct. Embryos were recovered from both systems at days 2, 3, 4, 5, 6, and 7. The results of this study confirm our previous observations (Rizos et al. 2002b, 2003) that the pattern of expression of several genes differs in blastocysts produced by culture *in vitro* or *in vivo*. More importantly however, the results demonstrate that these differences in expression are apparent at very early cleavage stages (in the case of Bax, SOX, Cx43, for example), in some cases after only 1 day of culture *in vitro*.

Concluding Remarks

From the above, it is clear that any improvement in oocyte developmental competence is likely to come from research focussed at the oocyte end of the oocyte–blastocyst developmental axis. In contrast, improvements in the quality of blastocysts produced *in vitro* will only occur through modification of the post-fertilization culture conditions. By altering the conditions of oocyte maturation and embryo culture, respectively, in such a way as to change the pattern of gene expression within the oocyte or embryo to mirror more closely that which occurs in their *in vivo* counterparts, it may be possible to produce, not only more blastocyst stage embryos, but more importantly, blastocysts of better quality.

References

Abd El Razek IM, Charpigny G, Kodja S, Marquant-Le Guienne B, Mermillod P, Guyader-Joly C, Humblot P, 2000: Differences in lipid composition between *in vivo*- and *in vitro*-produced bovine embryos. Theriogenology 53, 346 (abstract).

Bavister BD, Boatman DE, Leibfried ML, Loose M, Vernon MW, 1983: Fertilization and cleavage of rhesus monkey oocytes *in vitro*. Biol Reprod 28, 983–999.

Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Kominek DJ, Moyer AL, Famula TR, Sainz RD, Anderson GB, 2002: Morphology and morphometry of *in vivo*- and *in vitro*-produced bovine concepti from early pregnancy to term and association with high birth weights. Theriogenology 58, 973–994.

Besenfelder U, Havlicek V, Mosslacher G, Brem G, 2001: Collection of tubal stage bovine embryos by means of endoscopy: a technique report. Theriogenology 55, 837–845.

Besenfelder U, Brem G, 1998: Tubal transfer of bovine embryos: a simple endoscopic method reducing long-term exposure of *in vitro* produced embryos. Theriogenology 50, 739–745.

Blondin P, Coenan K, Guilbault LA, Sirard MA, 1997a: In vitro production of bovine embryos: developmental competence is acquired before maturation. Theriogenology 47, 1061–1075.

Blondin P, Guibault L, Sirard MA, 1997b: The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. Theriogenology 48, 803–813.

Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA, 2002: Manipulation of follicular development to produce developmentally competent bovine oocytes. Biol Reprod 66, 38–43.

Boni R, Tosti E, Roviello S, Dale B, 1999: Intracellular communication in *in vivo*- and *in vitro*-produced bovine embryos. Biol Reprod 61, 1050–1055.

Bordignon V, Morin N, Durocher J, Bousquet D, Smith LC, 1997: GnRH improves the recovery rate and the *in vitro* developmental competence of oocytes obtained by transvaginal follicular aspiration from superstimulated heifers. Theriogenology 48, 291–298.

Brevini-Gandolfi T, Lonergan P, Cillo F, Favetta LA, Fair T, Gandolfi F, 2002: Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. Mol Reprod Dev 63, 510–517.

Comizzoli P, Marquant-Le Guienne B, Heyman Y, Renard JP, 2000: Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes. Biol Reprod 62, 1677–1684.

Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE, 2000: Ultrastructural morphometry of bovine compact morulae produced *in vivo* or *in vitro*. Biol Reprod 62, 1459–1465.

Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE, 2001: Ultrastructural Morphometry of Bovine Blastocysts produced *in vivo* or *in vitro*. Biol Reprod 64, 1375–1385.

Crosier AE, Farin CE, Rodriguez KF, Blondin P, Alexander JE, Farin PW, 2002: Development of skeletal muscle and expression of candidate genes in bovine fetuses from embryos produced *in vivo* or *in vitro*. Biol Reprod 67, 401–408.

Dalbies-Tran R, Mermillod P, 2003: Use of heterologous complementary DNA screening to analyze bovine oocyte transcriptome and its evolution during *in vitro* maturation. Biol Reprod 68, 252–261.

De Sousa PA, Caveney A, Westhusin ME, Watson AJ, 1998a: Temporal patterns of embryonic gene expression and their dependence on oogenetic factors. Theriogenology 49, 115–128.

De Sousa PA, Westhusin ME, Watson AJ, 1998b: Analysis of variation in relative mRNA abundance for specific gene transcripts in single bovine oocytes and early embryos. Mol Reprod Dev 49, 119–130.

Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM, Vos PL, 2002: Effects of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology 57, 5–20.

Dinnyes A, Lonergan P, Fair T, Boland MP, Yang X, 1999: Timing of the first cleavage post-insemination affects cryosurvival of *in vitro*-produced bovine blastocysts. Mol Reprod Dev 53, 318–324.

Doherty AS, Mann MRW, Tremblay KD, Bartolomei MS, Schultz RM, 2000: Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol Reprod 62, 1526–1535.

Duby RT, Hill JL, O'Callaghan D, Overstrom EW, Boland MP, 1997: Changes induced in the bovine zona pellucida by ovine and bovine oviducts. Theriogenology 47, 332 (abstract).

Eckert J, Niemann H, 1998: mRNA expression of leukaemia inhibitory factor (Lif) and its receptor subunits glycoprotein 130 and Lif-receptor-beta in bovine embryos derived *in vitro* or *in vivo*. Molecular Human Reproduction **4**, 957–965.

Eid LN, Parrish JJ, 1995: Duration of G2-phase and onset of M-phase during the first cell cycle of the bovine embryo is dependent on bull *in vivo* fertility. Theriogenology **205**, (abstract).

Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP, 2000: Culture of *in vitro* produced bovine zygotes *in vitro* vs *in vivo*: implications for early embryo development and quality. Theriogenology **54**, 659–673.

Fair T, Hyttel P, Greve T, 1995: Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev **42**, 437–442.

Fair T, Lonergan P, Dinnyes A, Cottell D, Hyttel P, Ward FA, Boland MP, 2001: Ultrastructure of bovine blastocysts following cryopreservation: effect of method of embryo production on blastocyst quality. Mol Reprod Dev **58**, 186–195.

Fair T, Murphy M, Martin F, Boland M, Lonergan P, 2002: Identification of differentially expressed genes in early and late cleaving bovine embryos using suppressive subtractive hybridization. Theriogenology **57**, 636 (abstract).

Fenwick J, Platteau P, Murdoch AP, Herbert M, 2002: Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos *in vitro*. Hum Reprod **17**, 407–412.

Galli C, Lazzari G, 1996: Practical aspects of IVM/IVF in cattle. Anim Reprod Sci **42**, 371–379.

Gandolfi TA, Gandolfi F, 2001: The maternal legacy to the embryo: cytoplasmic components and their effects on early development. Theriogenology **55**, 1255–1276.

Goto T, Jones GM, Lolatgis N, Pera MF, Trounson A, 2002: Identification and characterisation of known and novel transcripts expressed during the final stages of human oocyte matutation. Mol Reprod Dev **62**, 13–28.

Greve T, Xu KP, Callesen H, Hyttel P, 1987: *In vivo* development of *in vitro* fertilized bovine oocytes matured *in vivo* versus *in vitro*. J IVF Embryo Transfer **4**, 281–285.

Grisart B, Massip A, Dessy F, 1994: Cinematographic analysis of bovne embryo development in serum-free oviduct-conditioned medium. J Reprod Fertil **101**, 257–264.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA, 1995: Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology **43**, 141–152.

Hendriksen PJM, Vos PLAM, Steenweg WNM, Bevers MM, Dieleman SJ, 2000: Bovine follicular development and its effect on the *in vitro* competence of oocytes. Theriogenology **53**, 11–20.

Holm P, Walker SK, Seamark RF, 1996: Embryo viability, duration of gestation and birth weight in sheep after transfer of *in vitro* matured and *in vitro* fertilized zygotes cultured *in vitro* or *in vivo*. J Reprod Fertil **107**, 175–181.

Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H, 1998: Developmental kinetics of the first cell cycles of bovine *in vitro* produced embryos in relation to their *in vitro* viability and sex. Theriogenology **50**, 1285–1299.

Holm P, Booth PJ, Callesen H, 2002: Kinetics of early *in vitro* development of bovine *in vivo*- and *in vitro*- derived zygotes produced and/or cultured in chemically defined or serum-containing media. Reproduction **123**, 553–565.

Holm P, Callesen H, 1998: *In vivo* versus *in vitro* produced bovine ova: similarities and differences relevant for practical application. 14th Meeting of the European Embryo Transfer Society, Venice.

Hunter RHF, Lawson RAS, Rowson LEA, 1972: Maturation, transplantation and fertilization of ovarian oocytes in cattle. J Reprod Fertil **30**, 325–328.

Hyttel P, Callesen H, Greve T, 1986a: Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. J Reprod Fertil **76**, 645–656.

Hyttel P, Xu KP, Smith S, Greve T, 1986b: Ultrastructure of *in vitro* oocyte maturation in cattle. J Reprod Fertil **78**, 615–625.

Hyttel P, Greve T, Callesen H, 1989: Ultrastructural aspects of oocyte maturation and fertilization in cattle. J Reprod Fert, Suppl. **38**, 35–47.

Hyttel P, Fair T, Callesen H, Greve T, 1997: Oocyte growth, capacitation and final maturation in cattle. Theriogenology **47**, 23–32.

Khurana NK, Niemann H, 2000: Energy metabolism in preimplantation bovine embryos derived *in vitro* or *in vivo*. Biol Reprod **62**, 847–856.

Knijn HM, Wrenzycki C, Hendriksen PJ, Vos PL, Herrmann D, Van Der Weijden GC, Niemann H, Dieleman SJ, 2002: Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived *in vitro* or *in vivo*. Reproduction **124**, 365–75.

Kruip TAM, den Daas JHG, 1997: *In vitro* produced and cloned embryos effects on pregnancy, parturition and offspring. Theriogenology **47**, 43–52.

Kubelka M, Motlik J, Schultz RM, Pavlok A, 2000: Butyrolactone I reversibly inhibits meiotic maturation of bovine oocytes without influencing chromosome condensation activity. Biol Reprod **62**, 292–301.

Kuran M, McEvoy TG, Young LE, Broadbent PJ, Robinson JJ, Sinclair KD, 2000: Ovine fetal development following different periods of *in vitro* culture. Theriogenology **53**, 275 (abstract).

Langendonckt AV, Donnay I, Schuurbiers N, Auquier P, Carolan C, Massip A, Dessy F, 1997: Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J Reprod Fertil **109**, 87–93.

Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C, 2002: Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. Biol Reprod **67**, 767–775.

Lee K-F, Chow JFC, Xu J-S, Chan STH, Ip S-M, Yeung WSB, 2001: A comparative study of gene expression in murine embryos developed *in vivo*, cultured *in vitro*, and cocultured with human oviductal cells using messenger ribonucleic acid differential display. Biol Reprod **64**, 910–917.

van de Leemput EE, Vos PLAM, Zeinstra EC, Bevers MM, der Weijden GC, Dieleman SJ, 1999: Improved *in vitro* embryo development using *in vivo* matured oocytes from heifers superiovulated with a controlled preovulatory LH surge. Theriogenology **52**, 335–349.

Leibfried-Rutledge ML, Critser ES, Eyestone WH, Northey DL, First NL, 1987: Development potential of bovine oocytes matured *in vitro* or *in vivo*. Biol Reprod **36**, 376–383.

Lequarre A-S, Feugang J-M, Malhomme O, Donnay I, Massip A, Dessy F, Van Langendonckt A, 2001: Expression of Cu/Zn and Mn superoxide dismutases during bovine embryo development: influence of *in vitro* culture. Mol Reprod Dev **58**, 45–53.

Lockwood GM, Muttukrishna S, Ledger WL, 1998: Inhibins and activins in human ovulation, conception and pregnancy. Hum Reprod Update **4**, 284–295.

Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I, 1994: Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture *in vitro*. Mol Reprod Dev **37**, 48–53.

Lonergan P, Khatir H, Carolan C, Mermillod P, 1997: Bovine blastocyst production *in vitro* after inhibition of oocyte meiotic resumption for 24 h. J Reprod Fertil **109**, 355–365.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP, 1999: Effect of time interval from insemination to first cleavage on the developmental characteristics, sex and pregnancy rates following transfer of bovine preimplantation embryos. J Reprod Fertil **117**, 159–167.

Lonergan P, Dinnyes A, Fair T, Yang X, Boland M, 2000a: Bovine oocyte and embryo development following meiotic inhibition with butyrolactone I. Mol Reprod Dev **57**, 204–209.

Lonergan P, Gutierrez-Adan A, Pintado B, Fair T, Ward F, de la Fuente JD, Boland M, 2000b: Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two- cell embryos and blastocysts produced *in vitro*. Mol Reprod Dev **57**, 146–152.

Lonergan P, Rizos D, Kingston M, Boland MP, 2002: Temporal sensitivity of bovine embryos to culture environment. 18th Annual Meeting of the European Embryo Transfer Association, Rolduc.

Lonergan P, Gutierrez-Adan A, Rizos D, Pintado B, de la Fuente J, Wade M, Boland MP, 2003: Relative messenger RNA abundance in bovine oocytes collected at the LH surge and matured *in vitro* or their counterparts matured *in vivo*. Theriogenology **59**, 493 (abstract).

McCaffrey C, McEvoy TG, Diskin MG, Gwazdauskas FC, Kane MT, Sreenan JM, 1991: Successful co-culture of 1-4 cell cattle ova to the morula or blastocyst stage. J Reprod Fertil **91**, 119–124.

McEvoy TG, Sinclair KD, Broadbent PJ, Goodhand KL, Robinson JJ, 1998: Post-natal growth and development of simmental calves derived from *in vivo* or *in vitro* embryos. Reprod Fertil Dev **10**, 459–464.

McEvoy TG, Sinclair KD, Young LE, Wilmut I, Robinson JJ, 2000: Large offspring syndrome and other consequences of ruminant embryo culture *in vitro*: relevance to blastocyst culture in human ART. Hum Fertil **3**, 238–246.

McEvoy TG, Robinson JJ, Sinclair KD, 2001: Developmental consequences of embryo and cell manipulation in mice and farm animals. Reproduction **122**, 507–518.

McKiernan SH, Bavister BD, 1994: Timing of development is a critical parameter for predicting successful embryogenesis. Hum Reprod **9**, 2123–2129.

Marquant-Le Guienne B, Gerard M, Solari A, Thibault C, 1989: *In vitro* culture of bovine egg fertilized either *in vivo* or *in vitro*. Reprod Nutr Dev **29**, 559–568.

Memli E, First NL, 2000: Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. Zygote **8**, 87–96.

Minami N, Sasaki K, Aizawa A, Miyamoto M, Imai H, 2001: Analysis of gene expression in mouse 2-cell embryos using fluorescein differential display: comparison of culture environments. Biol Reprod **64**, 30–35.

Myers MW, Zhang L, Stroud BK, Godke RA, 1992: Development of *in vitro*-matured bovine oocytes fertilized either *in vitro* or *in vivo*. Proceedings of the 12th Congress on Animal Reproduction, The Hague.

Negrin Pereira N, McEvoy TG, Staines ME, King ME, Broadbent PJ, Mackie K, Ranilla M, Robinson JJ, 1997: Effect of exposure to two different culture systems on the development and metabolism of *in vivo* derived sheep zygotes. Theriogenology **47**, 377 (abstract).

Newcomb R, Christie WB, Rowson LEA, 1978: Birth of calves after *in vivo* fertilisation of oocytes removed from follicles and matured *in vitro*. Vet Rec **102**, 461–462.

Pavlok A, Lucas-Hahn A, Niemann H, 1992: Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev **31**, 63–67.

Pincus G, Enzmann EV, 1935: The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. J Exp Med **62**, 665–675.

Pinyopummintr T, Bavister BD, 1994: Development of bovine embryos in a cell-free culture medium: effects of type of serum, timing of its inclusion and heat inactivation. Theriogenology **41**, 1241–1249.

Pocar P, Brevini TA, Perazzoli F, Cillo F, Modina S, Gandolfi F, 2001: Cellular and molecular mechanisms mediating the effects of polychlorinated biphenyls on oocyte developmental competence in cattle. Mol Reprod Dev **60**, 535–541.

Pollard JW, Leibo SP, 1994: Chilling sensitivity of mammalian embryos. Theriogenology **41**, 101–106.

Rief S, Sinowatz F, Stojkovic M, Einspanier R, Wolf E, Prelle K, 2002: Effects of a novel co-culture system on development, metabolism and gene expression of bovine embryos produced *in vitro*. Reproduction **124**, 543–56.

Rizos D, Lonergan P, Ward F, Boland M (2001). Effect of modification of synthetic oviduct fluid (SOF) composition on yield and quality of bovine blastocysts. 17th Scientific Meeting of the European Embryo Transfer Association, Lyon.

Rizos D, Fair T, Papadopoulos S, Boland M, Lonergan P, 2002a: Developmental, qualitative and ultrastructural differences between ovine and bovine embryos produced *in vivo* or *in vitro*. Mol Reprod Dev **62**, 320–327.

Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, la Fuente J, Gutierrez-Adan de A, 2002b: Analysis of differential mRNA expression between bovine blastocysts produced in different culture systems: Implications for blastocyst quality. Biol Reprod **66**, 589–595.

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

Rizos D, Gutierrez-Adan A, Perez-Garnalo S, de la Fuente J, Boland MP, Lonergan P, 2003: Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod **68**, 236–243.

Robert C, Barnes FL, Hue I, Sirard M-A, 2000: Subtractive hybridization used to identify mRNA associated with the maturation of bovine oocytes. Mol Reprod Dev **57**, 167–175.

Robert C, Gagne D, Bousquet D, Barnes FL, Sirard MA, 2001: Differential display and suppressive subtractive hybridization used to identify granulosa cell messenger RNA associated with bovine oocyte developmental competence. Biol Reprod **64**, 1812–1820.

Robert C, Hue I, McGraw S, Gagne D, Sirard MA, 2002: Quantification of cyclin B1 and p34(cdc2) in bovine cumulus-oocyte complexes and expression mapping of genes involved in the cell cycle by complementary DNA macroarrays. Biol Reprod **67**, 1456–1464.

Sakkas D, Shoukir Y, Chardonnens D, Bianchi PG, Campana A, 1998: Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum Reprod **13**, 182–187.

Shoukir Y, Chardonnens D, Campana A, Bischof P, Sakkas D, 1998: The rate of development and time of transfer play different roles in influencing the viability of human blastocysts. Hum Reprod **13**, 676–681.

Sinclair KD, McEvoy TG, Maxfield EK, Maltin CA, Young LE, Wilmut I, Broadbent PJ, Robinson JJ, 1999: Aberrant

fetal growth and development after *in vitro* culture of sheep zygotes. J Reprod Fertil 116, 177–186.
Sinclair KD, Young LE, Wilmut I, McEvoy TG, 2000: In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. Hum Reprod 15(Suppl. 5), 68–86.
Sirard MA, 2001: Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. Theriogenology 55, 1241–1254.
Slimane W, Heyman Y, Renard J-P, 2000: Assessing chromosomal abnormalities by FISH analysis in 2-cell bovine embryos derived from *in vitro* and *in vivo* fertilization. Theriogenology 53, 432 (abstract).
Sreenan J, 1970: *In vitro* maturation and attempted fertilization of cattle follicular oocytes. J Agric Sci Camb 75, 393–396.
Thompson JG, 2000: *In vitro* culture and embryo metabolism of cattle and sheep embryos – a decade of achievement. Anim Reprod Sci 60–61, 263–275.
Totey SM, Daliri M, Rao KBCA, Pawshe CH, Taneja M, Chillar RS, 1996: Differential cleavage and developmental rates and their correlation with cell numbers and sex ratios in buffalo embryos generated *in vitro*. Theriogenology 45, 521–533.
Trounson AO, Willadsen SM, Rowson LEA, 1977: Fertilization and development capability of bovine follicular oocytes matured *in vitro* and *in vivo* and transferred to the oviducts of rabbits and cows. J Reprod Fertil 51, 321–327.
Van Soom A, Vlaenderen IV, Mahmoudzadeh AR, Deluyker H, de Kruif A, 1992: Compaction rate of *in vitro* fertilized bovine embryos related to the interval from insemination to first cleavage. Theriogenology 38, 905–919.
Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H, Thomsen PD, 1999: A high proportion of bovine blastocysts produced *in vitro* are mixoploid. Biol Reprod 60, 1273–1278.
Viuff D, Greve T, Avery B, Hyttel P, Brockhoff PB, Thomsen PD, 2000: Chromosome aberrations in *in vitro*-produced bovine embryos at Days 2-5 post-insemination. Biol Reprod 63, 1143–1148.
Viuff D, Hendriksen PJM, Vos PLAM, Dieleman SJ, Bibby BM, Greve T, Hyttel P, Thomsen PD, 2001: Chromosomal abnormalities and developmental kinetics in *in vivo*-developed cattle embryos at days 2-5 after ovulation. Biol Reprod 65, 204–208.
van Wagtendonk-de Leeuw AM, Mullaart E, de Roos APW, Merton JS, den Daas JHG, Kemp B, Ruigh L, 2000: Effects of different reproduction techniques: AI, MOET or IVP, on health and welfare of bovine offspring. Theriogenology 53, 575–597.
Walker SK, Hartwich KM, Robinson JS, 2000: Long-term effects on offspring of exposure of oocytes and embryos to chemical and physical agents. Hum Reprod Update 6, 564–577.
Ward FA, Rizos D, Corridan D, Quinn K, Boland MP, Lonergan P, 2001: Paternal influence on the time of the first embryonic cleavage post insemination and the implications for subsequent bovine embryo development *in vitro* and fertility *in vivo*. Mol Reprod Dev 60, 47–55.
Warner CM, McElhinny AS, Wu L, Cieluch C, Ke X, Cao W, Tang C, Exley GE, 1998: Role of the Ped gene and apoptosis genes in control of preimplantation development. J Assist Reprod Genet 15, 331–337.
Watson AJ, 1992: The cell biology of blastocyst development. Mol Reprod Dev 33, 492–504.
Watson AJ, Sousa PD, Caveney A, Bancroft LC, Natale D, Urquhart J, Westhusin ME, 2000: Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. Biol Reprod 62, 355–364.
Wrenzycki C, Herrmann D, Carnwath JW, Niemann H, 1999: Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. Mol Reprod Dev 53, 8–18.
Wrenzycki C, Herrmann D, Korsawe K, Hadeler K-G, Niemann H, 2000: Relative abundance of specific mRNAs in bovine embryos produced *in vivo* or *in vitro* employing two different culture systems. Theriogenology 53, 415 (abstract).
Wrenzycki C, Herrmann D, Keskintepe L, Martins A, Jr, Sirisathien S, Brackett B, Niemann H, 2001: Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. Hum Reprod 16, 893–901.
Wrenzycki C, De Sousa P, Overstrom EW, Duby RT, Herrman D, Watson AJ, Niemann H, O'Callaghan D, Boland MP, 2000: Effects of superovulated heifer diet type and quantity on relative mRNA abundances and pyruvate metabolism in recovered embryos. J Reprod Fertil 118, 69–78.
Young LE, Sinclair KD, Wilmut I, 1998: Large offspring syndrome in cattle and sheep. Rev Reprod 3, 155–163.
Zeng F, Schultz RM, 2003: Gene expression in mouse oocytes and preimplantation embryos: use of suppressive subtractive hybridization to identify oocyte- and embryo-specific genes. Biol Reprod 68, 31–39.

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Evolution of mRNA Polyadenylation Between Oocyte Maturation and First Embryonic Cleavage in Cattle and Its Relation With Developmental Competence

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ABSTRACT In this study we analyzed the pattern of polyadenylation changes that takes place between the resumption of meiosis and the first cleavage of bovine oocytes. Moreover, we investigated whether the delayed occurrence of the first cleavage division, which characterizes embryos of low developmental competence, is accompanied by an altered polyadenylation pattern of individual transcripts. We determined the polyadenylation status of a group of genes that characterize physiological processes, involved in early differentiation (Oct-4), compaction, and cavitation (β-actin, plakophilin, connexin-32, connexin-43), energy metabolism (glucose transporter type 1, pyruvate dehydrogenase phosphatase), RNA processing (RNA poly(A) polymerase), and stress (heat shock protein 70). RNA was isolated from pools of 20 oocytes or embryos at the germinal vesicle (GV) stage, at the end of in vitro maturation, at the end of in vitro fertilization, and at the time of the first cleavage. Cleavage was assessed 27, 30, 36, 42 hr post insemination (hpi), and at the latter time the remaining uncleaved oocytes were retained as a group. Between oocyte isolation and first cleavage at 27 hpi (best quality embryos), the poly(A) tail of individual transcripts followed four patterns: no changes (β-actin, PDP); gradual reduction (Cx-43, Oct-4, Plako); gradual elongation (Cx-32, TPA); reduction followed by elongation (PAP, HSP-70, Glut-1). If the interval between insemination and first cleavage was longer than 27 hpi (progressively lower quality embryos) further changes of polyadenylation were observed, which differed for each gene considered. These data indicated that specific changes in polyadanylation contribute to the modulation of gene expression in bovine embryos at this stage of development. Defective developmental competence is accompanied by abnormal polyadenylation levels of specific maternal mRNAs with synchrony between polyadenylation and cleavage emerging as an apparently important factor. *Mol. Reprod. Dev. 63: 510–517, 2002.*

Key Words: mRNA polyadenylation; oocyte; embryo; bovine; development

INTRODUCTION

The time of the first embryonic cleavage post-insemination has major, long-lasting effects on the subsequent development of the bovine embryo to the blastocyst stage and is considered to be a good indicator of those most suitable for transfer to recipients (Yadav et al., 1993; Plante et al., 1994; van Soom et al., 1997; Dinnyes et al., 1999; Lonergan et al., 1999). This situation is not unique to the bovine embryo. A relationship between the kinetics of early embryonic cleavage and subsequent development has been observed in mice (Warner et al., 1998), hamsters (McKiernan and Bavister, 1994), *Rhesus* monkeys (Bavister et al., 1983; Morgan et al., 1990), buffaloes (Totey et al., 1996), and humans (Lelaidier et al., 1995; Sakkas et al., 1998; Shoukir et al., 1998). These observations suggest that whether or not an embryo will reach the blastocyst stage, is largely established at the two-cell stage. Therefore, the division of embryos on the basis of the interval between in vitro insemination and first cleavage provides a powerful experimental tool for studying the mechanisms determining embryonic developmental competence that is meaningful for many mammalian species.

During the early cleavage stages, embryonic development is supported by maternal mRNAs and proteins synthesized and stored during oogenesis. These stores

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remain critical during the entire interval between fertilization and the so-called maternal–embryonic transition (MET) when the transcriptional activity of the embryonic genome becomes fully functional. The length of this period of time depends on the species considered. In mammals it can occur as early as the late two-cell stage, as in mice, or later in development, at the four-cell stage in pigs, between the four- and eight-cell stage in humans, at the eight-cell stage in rabbits, and between the 8- and 16-cell stage in sheep and cattle (Telford et al., 1990). Moreover, the stored maternal information is also involved in development after the MET since it has been shown in mice that proteins synthesized from maternal mRNA are very stable throughout preimplantation development (Renard et al., 1994). Therefore, a long interval can intervene between the synthesis of maternal mRNA and its translation.

The extent of poly(A) tail at the 3′ end of mRNA transcripts has emerged as an important regulatory element for determining their stability. Thus, control of polyadenylation represents a key regulatory step in gene expression and is known to be important for early embryonic development both in mammals and in lower species (Wormington, 1993; Vassalli and Stutz, 1995; Brevini-Gandolfi and Gandolfi, 2001).

The aim of the present study was to characterize the changes of polyadenylation occurring between the resumption of meiosis and the first cleavage. We analyzed a set of genes, known to be stored in the ooplasm, and we investigated the possible relationship between the polyadenylation status of maternal mRNAs and embryo developmental competence, measured in terms of the time interval from insemination to first cleavage as previously described (Lonergan et al., 1999).

For this purpose we used the method originally described by Salles and Strickland (Salles and Strickland, 1995) which we have previously applied to bovine oocytes (Brevini-Gandolfi et al., 1999), that allows the accurate determination, in minute samples, of the polyadenylation status of any mRNA whose full sequence is known.

The genes analyzed in this study were chosen because they are known to be expressed from the immature oocyte stage to the blastocyst stage (Watson et al., 1992; Wrenzycki et al., 1998; Brevini-Gandolfi et al., 1999; van Eijk et al., 1999)—with the exception of PDP, for which information is limited to the oocyte stage—and, therefore, were considered to be suitable candidates for studying the storage process of mRNA molecules that will be used during early preimplantation development.

MATERIALS AND METHODS

In Vitro Production of Embryos

Chemicals were purchased from Sigma Chemical Co (St Louis, MO) unless otherwise indicated. A stock solution of 10 μg/ml epidermal growth factor (EGF) was prepared, divided into aliquots and stored at −20°C until use.

Cumulus oocyte complexes (COCs) were obtained by aspiration from follicles of ovaries from slaughtered cows. Following four washes in modified phosphate-buffered saline (PBS, supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamycin, and 0.5 mg/ml bovine serum albumin, Sigma fraction V, cat # A-9647), groups of approximately 50 COCs were placed in 500 μl of maturation medium for 24 hr culture at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was Medium 199 supplemented with 10% FCS and 10 ng EGF/ml.

For IVF, COCs were washed four times in PBS and then in fertilization medium before being transferred in groups of 50 into four-well plates containing 250 μl of fertilization medium (TALP, containing 10 μg/ml heparin, Calbiochem, San Diego, CA) per well. Frozen–thawed semen from a single ejaculate was used in all experiments. The semen was centrifuged on a Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (2 ml at 45% over 2 ml at 90%) for 20 min at 700*g*. Viable spermatozoa, collected at the bottom of the 90% fraction, were washed in TALP and pelleted by centrifugation at 100*g* for 10 min. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of TALP to give a concentration of 2×10^6 spermatozoa/ml; 250 μl of this suspension was added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/ml. Plates were incubated for 24 hr in 5% CO_2 in humidified air at 39°C.

Embryo culture was carried out in synthetic oviduct fluid (SOF) under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 (Carolan et al., 1995). Twenty-four hours after insemination, presumptive zygotes were denuded by vortexing for 2 min in 2 ml of PBS. The zygotes were subsequently washed four times in PBS and then in SOF before being transferred to the culture droplets (25 zygotes/25 μl medium).

Culture drops were examined at 27, 30, 33, 36, and 42 hr post insemination (hpi). At each time point, all cleaved embryos were removed, washed in PBS, placed in an Eppendorf tube, and snap frozen in liquid nitrogen. At 42 hpi, those presumptive zygotes that remained uncleaved were also snap frozen. In addition, a representative number of oocytes were frozen for RNA analysis at the time of their isolation from the follicle (GV stage), at the end of maturation (MII stage), and at the end of fertilization (presumptive zygotes, approximately 24 hpi). Cumulus cells were carefully removed before freezing.

RNA Extraction and PCR Poly-A Test

RNA was isolated from pools of 20 oocytes or embryos using the acid–phenol method according to Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). No additional carrier RNA was used as recommended by Salles and Strickland (Salles and Strickland, 1995).

Poly(A) tail length was determined as described before (Salles and Strickland, 1995), with some minor modifications.

Reverse transcription. RNA was denatured at 65°C for 5 min in a final volume of 7 μl, saturating the poly(A) tail with 25 ng of phosphorylated oligo(dT) [$p(dT)_{12-18}$] (Amersham Pharmacia Biotech, United Kingdom). Ligation of oligo(dT) [$p(dT)_{12-18}$] was then carried out at 42°C in 13 μl of a pre-warmed mix, consisting of 4 μl of sterile water, 10 U of T4 DNA ligase (Gibco BRL Life Technologies), 4 μl of 5X Superscript RNase H reverse transcriptase RT buffer (Gibco BRL Life Technologies), 1 μl of 10 mM dNTPs, 2 μl of 0.1 M DTT, and 1 μl 10 mM ATP. After 30 min of incubation, 200 ng of a specifically designed oligo(dT)-anchor primer were added and the temperature was lowered to 12°C. The oligo(dT)-anchor primer with nucleotide sequence 5′-GCGAGCTCCGCGGCCGCGT$_{12}$-3′ was ligated with a 2 hr incubation period to the extreme 3′ end of the poly(A) tail. Samples were then transferred back to 42°C. Reverse transcription was performed with 500 U Superscript RNase H-RT (Gibco BRL Life Technologies, United Kingdom) for 1 hr. Enzymes were inactivated with 30 min incubation at 70°C.

PCR amplification. The PCR Poly-A test requires the use of primers located close to the 3′ end (within 400 nucleotides) in order to provide the best PCR product size resolution (Salles and Strickland, 1995); therefore, the choice of genes was limited to those whose sequence was fully known in the bovine species. Primer sequences and accession numbers of the selected genes are specified in Table 1.

PCR runs were carried out with oligo(dT)-anchor and 5′ primers designed as above. One microlitre of the poly (A)-cDNA product was amplified in a reaction mix consisting of 3 μl of $MgCl_2$ (25 mM), 0.3 μl of Taq polymerase (5 units/μl), 2 μl of 10× PCR buffer (Boehringer Mannheim, I), 1 μl of 10 mM dNTPs, and 10.2 μl of sterile water. To spike the reaction 5 μCi of ^{32}P-dATP (specific activity 10 mCi/ml) were added to the mix. PCR was carried out in an automated thermal cycler (Perkin Elmer, Cetus Instruments, USA), using the following conditions: 30 sec at 93°C (double strand denaturation temperature), 1 min at 61°C (annealing temperature), 1 min at 72°C (double strand extension temperature), performing 38 cycles. The amplified products were purified using Qiaquick PCR purification spin columns (Qiagen, Inc., USA) and separated on 3.5% TBE polyacrylamide gels (Sambrook et al., 1989) with a labeled 50 bp DNA ladder (Gibco BRL Life Technologies, United Kingdom) in order to allow size determination. Gels were exposed overnight to Amersham Hyperfilm β-max. The identity of each fragment was confirmed by restriction enzyme analysis, with specific endonucleases known to cut within the region amplified by the PCR primers used.

Statistical analysis. Size detection of each band in each gel was carried out using Epson GT-8000 Scanner; transcript length of each three replicates was then determined with GEL 1.01 as described by Lacroix (1994). Data obtained were subjected to statistical analysis using SuperAnova v. 1.11 (Abacus Concepts, Inc.).

RESULTS

The entire set of genes taken into consideration was studied in each pool of 20 oocytes or embryos and analysis was repeated on at least three different pools for each time point considered. No statistically significant variations between replicates among the three pools were observed.

Two aspects of mRNA polyadenylation were studied.

Changes of Poly(A) Tail Length Between GV Stage and First Cleavage

In this set of experiments poly(A) tail length was examined at the time of oocyte isolation from the follicle (GV stage), at the end of in vitro maturation (metephase II stage), at the end of in vitro fertilization, (presumptive zygote stage), and at the time of the first cleavage, 27 hr after insemination (two-cell stage). The results obtained are shown in Figure 1 and Table 2.

As described previously (Brevini-Gandolfi et al., 1999) at the metaphase II (MII) stage poly(A) tail was significantly shorter than at the GV stage in many of the examined transcripts with only a few exceptions: β-actin and PDP, whose transcripts remained of the same length; TPA and Cx 32, whose transcripts elongated during IVM. In most cases, poly(A) tail modifications followed the same trend also at the subsequent stages: i.e., β-actin and PDP remained unchanged at the presumptive zygote and two-cell stages; Cx-43, OCT-4 and Plako became progressively shorter, while Cx-32 and TPA gradually increased their poly(A) tail. However, this increase of polyadenylation was not statistically significant for TPA. Some transcripts showed a

TABLE 1. List of Examined Genes and Primers Used for PCR Poly-A Test of mRNA in Bovine Oocytes/Embryos

Target gene	Sequence 5′–3′	Position	Accession number
β-actin	TGAACCCTAAGGCCAACCGTG	1009–1029	GenBank U02295
Connexin 43 (Cx 43)	ACAGTCTTTTGGAGTAACCAGCA	2526–2548	EMBL J05535
Connexin 32 (Cx 32)	AGTGGAGGGAGGGTACTGT	1368–1386	EMBL X95311
Tissue type plasminogen activator (TPA)	AAAGCATGTCTCAGTAGGCA	1992–2011	EMBL X85800
Glucose transporter type 1 (Glut-1)	ATTAACCTTTCCTTGCCTGAGAC	2193–2215	EMBL M6044
Heat shock protein 70 (HSP70)	GAAGAAGGTGCTGGACAAGTG	1857–1877	EMBL U09861
Oct-4	GTGAGAGGCAACCTGGAGAG	721–740	GenBank F022988
Plakophilin (Plako)	ACACTGTGAGGAACCTGATGG	2073–2093	EMBL Z37975
Pyruvate dehydrogenase phosphatase (PDP)	ATTACACACCTCCTTATCTCACTGC	1330–1354	EMBL L18966
RNA poly(A) polymerase (PAP)	TAGGCCAGCCACATTAATCTCTA	2951–2973	EMBL X63436

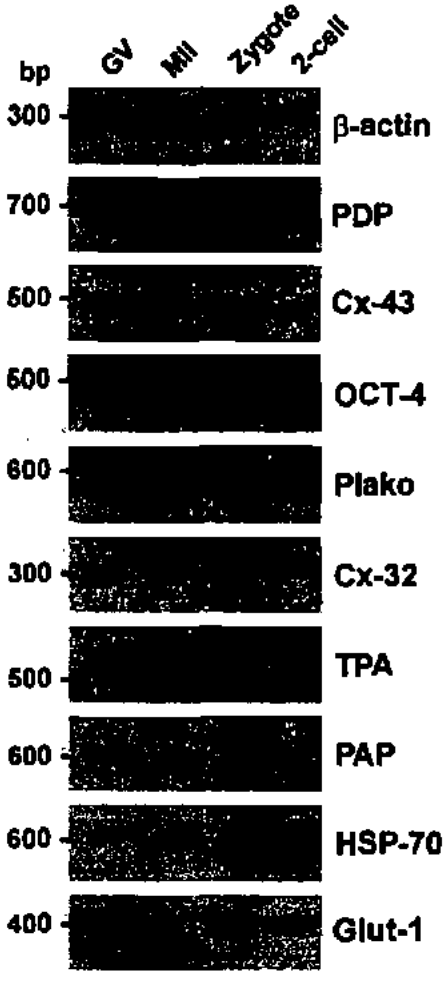

Fig. 1. Changes in polyadenylation level of mRNAs extracted from bovine oocytes at the germinal vesicle (GV) stage, oocytes at the metaphase II (MII) stage, putative zygotes at the end of IVF (zygote), and two-cell embryos cleaved 27 hpi (2-cell). ^{32}P-labelled amplification products were run on 3% TBE polyacrylamide gels after mRNAs were reverse transcribed and amplified using specific primers for each gene (see Materials and Methods and Table 1 for details). The left-hand column shows the appropriate molecular weight marker for each gene (bp).

different pattern: PAP poly(A) tail became shorter at the zygote stage and was longer at the two-cell stage; HSP-70 poly(A) tail also became shorter during IVF but no further changes were visible at the two-cell stage; Glut-1 poly(A) tail became shorter during IVF but significantly increased at the time of the first cleavage.

Differences of Poly(A) Tail Length in Relation to Embryo Developmental Competence

In this case we examined the poly(A) tail length of transcripts isolated from embryos that reached the two-cell stage at different time intervals from insemination (27, 30, 36, and 42 hpi); presumptive zygotes that did not cleave after 42 hpi were also included (n.c.). A representative pattern for each transcript is shown in Figure 2 and the results obtained after statistical analysis are presented in Table 3.

As observed in the previous experiment, the poly(A) tails of β-actin and PDP mRNA molecules did not change their length irrespective of time of first cleavage.

In contrast, the poly(A) tail length of all other transcripts changed in relation to the interval occurring between insemination and first cleavage. Cx-43, OCT-4, and Plako transcripts showed a progressively shorter poly(A) tail, leaving unchanged the trend observed between GV and cleavage. The same was observed for Cx-32 and TPA transcripts, with the only difference being that, in this case, the trend was towards an increase of polyadenylation. Moreover, this increase became statistically significant when the time interval from insemination was 36 hr for TPA and 42 hr for Cx-32.

PAP, HSP-70, and Glut-1 showed another different pattern. The poly(A) tail of all three became shorter during IVM, and then began to elongate, either at fertilization (Glut-1), at 27 hpi (PAP), or at 30 hpi (HSP-70). From these time points onwards the three transcripts showed a progressive increase of polyadenylation.

Finally, the degree of polyadenylation found in the presumptive zygotes that did not cleave by 42 hpi was different from that of their cleaved counterparts, as well

TABLE 2. Poly(A) Tail Difference in mRNA From Bovine Oocytes at the Germinal Vesicle (GV) Stage, Oocytes at the Metaphase II (MII) Stage, Putative Zygotes at the End of IVF (Zygote), and Two-Cell Embryos Cleaved 27 hpi (Two-cell)

	GV	MII	Zygote	Two-cell
βactin	230 ± 8.16^a	247 ± 16.5^a	260 ± 15.3^a	267 ± 12.2^a
PDP	670 ± 7.62^a	662 ± 7.04^a	664 ± 5.50^a	672 ± 6.55^a
Cx-43	508 ± 6.18^a	492 ± 6.12^b	471 ± 4.11^c	450 ± 3.27^d
OCT-4	509 ± 5.31^a	485 ± 3.66^b	460 ± 2.86^c	437 ± 3.66^d
Plako	642 ± 4.90^a	625 ± 4.92^b	591 ± 3.25^c	548 ± 5.31^d
Cx-32	274 ± 4.11^a	299 ± 3.26^b	347 ± 4.50^c	366 ± 5.31^d
TPA	510 ± 5.18^a	540 ± 6.13^b	557 ± 4.92^c	582 ± 4.99^c
PAP	592 ± 5.71^a	569 ± 4.91^b	546 ± 4.11^c	582 ± 2.87^d
HSP-70	612 ± 7.51^a	569 ± 3.26^b	539 ± 3.68^c	550 ± 4.50^c
Glut-1	352 ± 5.71^a	321 ± 2.86^b	368 ± 5.32^c	395 ± 3.49^d

Numbers shown in the table are transcript length (expressed as base pairs) and represent the mean ± standard deviation of the three replicates obtained for each gene considered at the different stages. Statistical analysis was carried out using Super Anova 1.11. Different superscript letters indicate significant difference ($P < 0.05$).

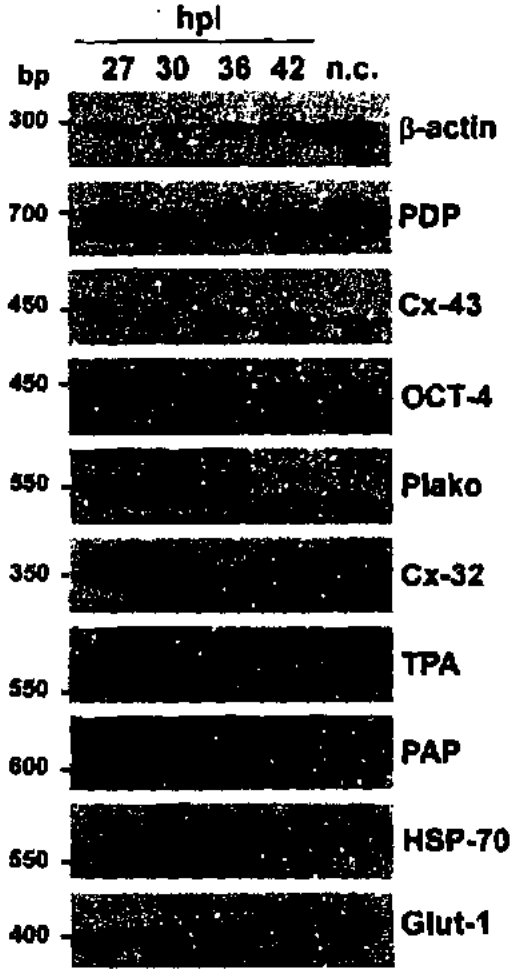

Fig. 2. Differences in the poly(A) tail length of mRNAs isolated from bovine two-cell embryos cleaved at different times after insemination (hpi) and from oocytes that had not cleaved by 42 hpi (n.c.). ^{32}P-labelled amplification products were separated on 3% TBE polyacrylamide gels following reverse transcription and PCR amplification using specific primers for each gene (see Materials and Methods and Table 1 for details). The left-hand column shows the appropriate molecular weight marker for each gene (bp).

as from that observed at the other stage of development, including the mature oocytes and the presumptive zygotes analyzed 24 hpi. The only exceptions were β-actin and PDP whose polyadenylation level remained unchanged throughout the experiment, as described above. The poly(A) tail of all the other transcripts was either longer or shorter depending on the trend shown by the embryos that cleaved between 27 and 42 hpi.

DISCUSSION

Translational control is very important in early embryos since they are commonly transcriptionally inactive, or nearly so, yet require rapid changes in the proteins they contain, in order to regulate key developmental decisions. These processes are very well studied in lower organisms such as *Xenopus* and *Drosophila*, as well as in mouse, but little is known of how they are conserved in other mammalian species and of the possible relationship between translational control and developmental competence. Though several mechanisms are involved in the control of translation, sequences in the 3′ untranslated region and the poly(A) tail can have dramatic effects on initiation frequency, with particularly profound effects in oogenesis and early development (Gray and Wickens, 1998). However, apart from some recent data obtained in mouse (Oh et al., 2000), very little is known about how many maternal mRNA molecules are translationally controlled by polyadenylation. Therefore, in the present report we investigated two different aspects of maternal mRNA behavior in cattle oocytes and embryos: firstly, how the levels of polyadenylation change between the beginning of meiotic resumption and the first cleavage, and secondly, how such changes may be related to embryo developmental competence. To address these questions we used the PCR PolyA Test for analyzing the polyadenylation levels of a set of genes known to be stored in the ooplasm, to be expressed from oocyte maturation to the blastocyst stage, and to be representative of many different processes taking place during early development (Brevini-Gandolfi et al., 1999).

TABLE 3. Changes in Poly(A) Tail of mRNAs Obtained From Bovine Two-Cell Embryos Cleaved at Different Times After Insemination (hpI) and From Ooctyes That Have Not Cleaved by 42 hpi (nc)

	hpi				
	27 hr	30 hr	36 hr	42 hr	n.c.
β-actin	280 ± 3.13^{a}	282 ± 4.58^{a}	285 ± 4.52^{a}	287 ± 3.82^{a}	278 ± 3.51^{a}
PDP	671 ± 7.35^{a}	682 ± 2.62^{a}	691 ± 7.76^{a}	689 ± 7.35^{a}	684 ± 12.39^{a}
Cx-43	455 ± 4.51^{a}	441 ± 3.68^{b}	422 ± 2.98^{c}	405 ± 4.04^{d}	392 ± 5.72^{e}
OCT-4	433 ± 6.55^{a}	403 ± 4.92^{b}	372 ± 8.02^{c}	338 ± 3.60^{d}	300 ± 8.54^{e}
Plako	542 ± 5.31^{a}	509 ± 4.94^{b}	485 ± 7.35^{c}	459 ± 3.27^{d}	424 ± 4.55^{e}
Cx-32	361 ± 4.92^{a}	$366 \pm 6.55^{a/b}$	$376 \pm 0.94^{b/c}$	384 ± 5.31^{c}	413 ± 4.92^{d}
TPA	586 ± 4.92^{a}	580 ± 3.27^{a}	607 ± 3.86^{b}	647 ± 4.64^{c}	671 ± 3.27^{d}
PAP	590 ± 6.95^{a}	626 ± 4.53^{b}	651 ± 6.55^{c}	670 ± 6.94^{d}	709 ± 4.95^{e}
HSP-70	553 ± 4.08^{a}	584 ± 4.92^{b}	602 ± 4.99^{c}	635 ± 4.18^{d}	672 ± 2.87^{e}
Glut-1	393 ± 4.19^{a}	418 ± 6.53^{b}	436 ± 2.05^{c}	465 ± 0.82^{d}	496 ± 7.35^{e}

Numbers shown in the table are transcript length (expressed as base pairs) and represent the mean ± standard deviation measured in three replicates for each gene at different hpi. Statistical analysis was carried out using SuperAnova 1.11. Different superscript letters denote statistical difference ($P < 0.05$).

The results obtained indicate that, in cattle, mRNA polyadenylation changes during this period with four different patterns depending on the transcript examined.

Cx-43, Oct-4, and Plako became progressively less polyadenylated, following what is described as the default pattern in *Xenopus* and mouse (Hake and Richter, 1997). The total amount of Cx-43 in bovine oocytes and embryos is known to remain constant between the onset of meiosis and the 2–4-cell stage (Wrenzycki et al., 1999), but the complete disappearance of Cx-43 positive gap junctions between cumulus cells and the oocyte occurs after 9 hr of maturation (Sutovsky et al., 1993). Therefore, the modulation of Cx-43 expression with no detectable mRNA loss may be explained by the progressive deadenylation that is usually associated with translational inactivation.

Cx-32 and TPA became more polyadenylated following the other well established pattern described in other species (Seydoux, 1996). Poly(A) tail extension is known to activate translation (Richter, 1996) and, consistent with the observations presented here, Cx-32 begins to be detected in bovine oocytes cultured for 6 hr and its amount increases progressively up to 24 hr of maturation (Sutovsky et al., 1993). No specific data are available for TPA expression in cattle; however, this gene is a well-studied paradigm of dormant mRNA activation during mouse meiotic maturation (Vassalli et al., 1989).

While the previous two patterns are well characterized, in other species, the lack of any change in polyadenylation of PDP and β-actin transcripts, is more unusual and has been described before in cattle (Brevini-Gandolfi et al., 1999; Pocar et al., 2000) and, for β-actin and Melk, in mouse (Oh et al., 2000). The available data indicate that polyadenylation levels of these transcripts remain unchanged during the initial phase of development and are not related to different levels of developmental competence (see also below). This may reflect the fact that these genes are considered as housekeeping genes (Bilodeau-Goeseels and Schultz, 1997; Brevini-Gandolfi et al., 1999) and their translation efficiency, presumably, remains unchanged.

Finally PAP, HSP-70 and Glut-1 transcripts, which follow the default pattern of progressive deadenylation during oocyte maturation, showed a more or less gradual inversion becoming progressively more polyadenylated during the following stages. Increases in poly(A) tail length generally correlate with increases in translation, and decreases correlate with repression (Richter, 1996; Wickens et al., 1997). During the interval between oocyte maturation and full embryonic genome activation a steady decrease of maternal mRNA content is observed, though the degradation rate of individual messengers can vary according to the mRNA species (Paynton et al., 1988; Lequarre et al., 1997). Degradation of maternal transcripts is not necessarily accompanied by their deadenylation but a progressive increase of the poly(A) tail or no changes at all can occur at the same time. This suggests the presence of a regulatory loop for the fine tuning of gene expression. In this hypothesis the sharp decrease of Glut-1 total mRNA amount is accompanied by the elongation of its poly(A) tail which makes the remaining molecules more available for translation, but during the more gradual degradation of β-actin mRNA, no polyadenylation changes take place, and its translation efficiency, presumably, remains stable.

The bovine oocytes used to generate the embryos used in this experiment originated from ovaries collected at the abattoir and may constitute an extremely heterogeneous form of material (Gordon, 1994). We previously demonstrated a link between ovarian morphology and oocyte quality so that it is possible to segregate oocytes with high and low developmental competence (Gandolfi et al., 1997). Using this method we showed that poor developmental competence is associated with a defective (generally lower) polyadenylation level of maternal transcripts (Brevini-Gandolfi et al., 1999). Such a difference was present already at the time when the oocytes were isolated from the follicles and was not eliminated by the process of in vitro maturation. More recently it was possible to demonstrate that polyadenylation of maternal transcripts is also altered when bovine oocytes are exposed in vitro to PCBs (Pocar et al., 2001a). These are a mixture of chemicals responsible for widespread environmental contamination and affect oocyte maturation, fertilization, and embryonic developmental competence in cattle (Pocar et al., 2001b). In the present study, oocytes were selected using all the parameters known to indicate maximum developmental competence but, nevertheless, as demonstrated previously (Lonergan et al., 1999), the quality of about one third of them was below average. The data presented in this paper indicate that a lower developmental competence is accompanied by an altered pattern of maternal transcripts polyadenylation, confirming that, irrespectively of the cause, a link exists between reduced developmental competence and altered polyadenylation patterns.

Delayed cleavage appeared to be accompanied by different gene-specific polyadenylation changes: a progressive shortening of the poly(A) tail (Cx-43, Oct-4, and Plako), a progressive elongation (Cx-32, TPA, PAP, HSP-70, and Glut-1) as well as a lack of effect (β-actin and PDP). The significance of such alterations is, at present, unclear.

Previous reports have shown that the total amount of specific mRNA transcripts is altered in defective bovine oocytes and embryos (Lequarre et al., 1997; Wrenzycki et al., 1999; Lonergan et al., 2000). Moreover defective oocyte developmental competence and altered polyadenylation patterns have been described both in mice (Paynton et al., 1988) and cattle (Brevini-Gandolfi et al., 1999). Therefore, maternal mRNA degradation and polyadenylation are both affected in developmentally defective oocytes and embryos, but their relationship is unclear. It has been reported that HSP maternal transcripts are degraded more rapidly in embryos cultured in suboptimal conditions than in their normal counterparts (Wrenzycki et al., 1999). In our experiment,

defective embryos showed an elongated HSP-70 poly(A) tail, making this transcript more available to translation as a possible response to critical conditions. However this may not be the only possible pattern: PAP transcripts are more abundant in defective than in normal embryos (Wrenzycki et al., 1999) but we observed a longer poly(A) tail in the embryos of lesser quality. Moreover no differences were observed in the mRNA amounts of Cx-43, Plako, and Glut-1 between high and low quality embryos (Wrenzycki et al., 1999) but Cx-43 and Plako transcripts had a shorter poly(A) tail in low quality embryos while Glut-1 molecules were more polyadenylated.

Taken together these data indicate that poly(A) tail variation depends on the gene considered but it is difficult to establish a general pattern because, in our experiments, stability, elongation, and shortening were all observed. Previous observations limited to oocyte maturation in mice (Paynton et al., 1988) and cattle (Brevini-Gandolfi and Gandolfi, 2001) suggested that in the low developmental competence groups most of these changes occurred on a delayed schedule.

However, the data presented here suggest that, although changes in the level of polyadenylation are time-related, they cannot be defined as delayed. In fact a prolonged interval between fertilization and cleavage did not delay the changes of polyadenylation but rather these progressed irrespective of the cell cycle. Thus, it was possible to observe that maternal mRNA transcripts of severely delayed embryos (examined 42 hpi) became either more or less adenylated than their best quality counterpart (examined at 27 hpi) following the trend initiated at the end of maturation or at fertilization. The end result appears to he an asynchrony between developmental stage and polyadenylation levels.

In conclusion, since transcription is virtually absent in bovine embryos during the early stages of development, gene expression seems to be modulated not only by the different degradation rate of individual transcripts but also by their specific changes in polyadenylation. Similarly, poor developmental competence is accompanied by altered amounts of specific maternal mRNA molecules as well as by their abnormal polyadenylation levels with synchrony between polyadenylation and cleavage emerging as a new, apparently important factor.

REFERENCES

Bavister BD, Leibfried ML, Lieberman G. 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol Reprod 28:235–247.

Bilodeau-Goeseels S, Schultz GA. 1997. Changes in the relative abundance of various housekeeping gene transcripts in in vitro-produced early bovine embryos. Mol Reprod Dev 47:413–420.

Brevini-Gandolfi TAL, Gandolfi F. 2001. The maternal legacy to the embryo: Cytoplasmic components and their effects on early development. Theriogenlogy 55:1255–1276.

Brevini-Gandolfi TAL, Favetta LA, Mauri L, Luciano AM, Cillo F, Gandolfi F. 1999. Changes in poly(A) tail length of maternal transcripts during in vitro maturation of bovine oocytes and their relation with developmental competence. Mol Reprod Dev 52:427–433.

Carolan C, Lonergan P, Vanlangendonckt A, Mermillod P. 1995. Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in vitro. Theriogenology 43:1115–1128.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochim 162:156–159.

Dinnyes A, Lonergan P, Fair T, Boland MP, Yang X. 1999. Timing of the first cleavage post-insemination affects cryosurvival of in vitro-produced bovine blastocysts. Mol Reprod Dev 53:318–324.

Gandolfi F, Luciano AM, Modina S, Ponzini A, Pocar P, Armstrong DT, Lauria A. 1997. The in vitro developmental competence of bovine oocytes can be related to the morphology of the ovary. Theriogenology 48:1153–1160.

Gordon I. 1994. Laboratory production of cattle embryos. Wallingford: CAB International. pp 640.

Gray NK, Wickens M. 1998. Control of translation initiation in animals. Annu Rev Cell Dev Biol 14:399–458.

Hake LE, Richter JD. 1997. Translational regulation of maternal mRNA. Biochim Biophys Acta 1332:M31–M38.

Lacroix JM. 1994. DNA fragment size determination on agarose gel by using the application GEL. Comput Appl Biosci 10:185–187.

Lelaidier C, de Ziegler D, Freitas S, Olivennes F, Hazout A, Frydman R. 1995. Endometrium preparation with exogenous estradiol and progesterone for the transfer of cryopreserved blastocysts. Fertil Steril 63:919–921.

Lequarre AS, Grisart B, Moreau B, Schuurbiers N, Massip A, Dessy F. 1997. Glucose metabolism during bovine preimplantation development: Analysis of gene expression in single oocytes and embryos. Mol Reprod Dev 48:216–226.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP. 1999. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. J Reprod Fertil 117:159–167.

Lonergan P, Gutierrez-Adan A, Pintado B, Fair T, Ward F, Fuente JD, Boland M. 2000. Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro. Mol Reprod Dev 57:146–152.

McKiernan SH, Bavister BD. 1994. Timing of development is a critical parameter for predicting successful embryogenesis. Hum Reprod 9:2123–2129.

Morgan PM, Boatman DE, Bavister BD. 1990. Relationships between follicular fluid steroid hormone concentrations, oocyte maturity, in vitro fertilization and embryonic development in the Rhesus monkey. Mol Reprod Dev 27:145–151.

Oh B, Hwang SY, McLaughlin J, Solter D, Knowles BB. 2000. Timely translation during the mouse oocyte-to-embryo transition. Development 127:3795–3803.

Paynton BV, Rempel R, Bachvarova R. 1988. Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. Dev Biol 129:304–314.

Plante L, Plante C, Shepherd DL, King WA. 1994. Cleavage and H-3-uridine incorporation in bovine embryos of high in vitro developmental potential. Mol Reprod Dev 39:375–383.

Pocar P, Brevini-Gandolfi TAL, Navarrete-Santos A, Perazzoli F, Cillo F, Lauria A, Fischer B, Gandolfi F. 2000. Mechanisms of PCBs toxicity during in vitro maturation of bovine oocytes. Theriogenology 53:465.

Pocar P, Brevini TA, Perazzoli F, Cillo F, Modina S, Gandolfi F. 2001a. Cellular and molecular mechanisms mediating the effects of polychlorinated biphenyls on oocyte developmental competence in cattle. Mol Reprod Dev 60:535–541.

Pocar P, Perazzoli F, Luciano AM, Gandolfi F. 2001b. In vitro reproductive toxicity of polychlorinated biphenyls: Effects on oocyte maturation and developmental competence in cattle. Mol Reprod Dev 58:411–416.

Renard JP, Baldacci P, Richoux DV, Pournin S, Babinet C. 1994. A maternal factor affecting mouse blastocyst formation. Development 120:797–802.

Richter JD. 1996. Dynamics of poly(A) addition and removal during development. In: Hershey JWB, Mathews MB, Sonenberg N, editors. Translational control. New York: Cold Spring Harbor Laboratory Press. pp 481–503.

Sakkas D, Shoukir Y, Chardonnens D, Bianchi PG, Campana A. 1998. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum Reprod 13:182–187.

Salles FJ, Strickland S. 1995. Rapid and sensitive analysis of mRNA polyadenylation states by PCR. PCR Methods Appl 4:317–321.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning a laboratory manual. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press. pp 173–185.

Seydoux G. 1996. Mechanisms of translational control in early development. Curr Opin Genet Dev 6:555–561.

Shoukir Y, Chardonnens D, Campana A, Bischof P, Sakkas D. 1998. The rate of development and time of transfer play different roles in influencing the viability of human blastocysts. Hum Reprod 13:676–681.

Sutovsky P, Flechon JE, Flechon B, Motlik J, Peynot N, Chesne P, Heyman Y. 1993. Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes. Biol Reprod 49:1277–1287.

Telford NA, Watson AJ, Schultz GA. 1990. Transition from maternal to embryonic control in early mammalian development: A comparison of several species. Mol Reprod Dev 26:90–100.

Totey SM, Daliri M, Appa Rao KBC, Pawshe CH, Taneja M, Chillar RS. 1996. Differential cleavage and development rates and their correlation with cell numbers and sex ratios in buffalo embryos generated in vitro. Theriogenology 46:909–910.

van Eijk MJT, van Rooijen MA, Modina S, Scesi L, Folkers G, van TH, Bevers MM, Fisher SR, Lewin HA, Rakacolli D, Galli C, de VC, Trounson AO, Mummery CL, Gandolfi F. 1999. Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. Biol Reprod 60:1093–1103.

van Soom A, Ysebaert MT, de KA. 1997. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. Mol Reprod Dev 47:47–56.

Vassalli JD, Stutz A. 1995. Translational control. Awakening dormant mRNAs. Curr Biol 5:476–479.

Vassalli JD, Huarte J, Belin D, Gubler P, Vassalli A, O'Connell ML, Parton LA, Rickles RJ, Strickland S. 1989. Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes. Genes Dev 3:2163–2171.

Warner CM, Exley GE, McElhinny AS, Tang C. 1998. Genetic regulation of preimplantation mouse embryo survival. J Exp Zool 282: 272–279. [pii]

Watson AJ, Hogan A, Hahnel A, Wiemer KE, Shultz GA. 1992. Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. Mol Reprod Dev 31:87–95.

Wickens M, Anderson P, Jackson RJ. 1997. Life and death in the cytoplasm—messages from the 3' end. Curr Opin Genet Dev 7:220–232.

Wormington M. 1993. Poly(A) and translation: Development control. Cur Opin Cell Biol 5:950–954.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. 1998. Expression of RNA from developmentally important genes in preimplantation bovine embryos produced in TCM supplemented with BSA. J Reprod Fertil 112:387–398.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. 1999. Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. Mol Reprod Dev 53:8–18.

Yadav BR, King WA, Betteridge KJ. 1993. Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated invitro. Mol Reprod Dev 36:434–439.

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USE OF SEMEN FROM A BULL HETEROZYGOUS FOR THE 1/29 TRANSLOCATION IN AN IVF PROGRAM

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ABSTRACT

In cattle, a translocation of the Robertsonian type between the largest and smallest chromosome leads to a reduction in fertility. This is substantiated by reduced nonreturn rates in daughter groups of bulls carrying the 1/29 translocation and in the heterozygous bulls themselves. This reduction in fertility is thought to be due to the early death of embryos with unbalanced karyotypes. The influence of semen from a bull known to be heterozygous for the 1/29 translocation on the outcome of a bovine IVF program was investigated. There was a significant difference ($P<0.005$) in terms of cleavage rate (59.8 vs 71.1%) and blastocyst rate (12.0 vs 20.0%) between the carrier and control bull, respectively. There was no difference in blastocyst quality as measured by cell number. The results observed in vitro are consistent with the field fertility records of the 2 bulls in terms of nonreturn rates (59.2 vs 70.6%, for the carrier and control bull, respectively).

Key words: 1/29 translocation, bovine, oocyte fertilization, culture

INTRODUCTION

The transfer of a chromosome segment from its normal position to a position in a different chromosome is termed a translocation (23). The exchange of two entire long arms of one-armed chromosomes to form a single biarmed chromosome and thereby reducing the chromosome number is known as a Robertsonian translocation or centric fusion (23). In cattle, a translocation of this type involving the largest (No. 1) and the smallest (No. 29) chromosomes has been identified by conventional staining and banding techniques.

Observation of the 1/29 translocation in cattle was first reported by Gustavsson and Rockborn (9) in the Swedish Red and White Breed, and was followed in 1966 with an investigation of the general dairy cattle population of Sweden (4). That same year, the translocation was described in Norway (1), in the United States (10), and in the late sixties, in Italy (25) and Germany (24). In subsequent years, findings of the 1/29 translocation have been reported in many countries and various cattle breeds throughout the world. Popescu and Pech (21) presented a worldwide reference list of 231 articles published between 1964 and 1990 on the 1/29 translocation in cattle.

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Gustavsson (5) found that daughters of translocation sires returned to service more often than daughters of normal sires and it was suggested that this was due to an increased rate of embryonic death. Gustavsson (6) found a relatively high culling rate in daughters of bulls with autosomal translocation of the 1/29 type, and it was subsequently observed in a study of the chromosomes of 263 repeat breeder heifers that 31% showed the 1/29 translocation (7), thus confirming that daughters of bulls carrying the translocation have lowered fertility. Refsdal (22), working with cattle of the Norwegian Red Cattle breed, found that daughters of bulls heterozygous for the translocation showed a lower nonreturn rate than control animals, indicating a higher frequency of losses of fetuses in these animals as reported by Gustavsson (5). The difference in nonreturn percentages between the 2 groups increased rapidly from 0 to 30 up to 60 to 90 d, indicating that most fetal losses occured during this period (22).

Further evidence of the reduced fertility of carrier bulls came from the report of Dyrendahl and Gustavsson (3) who found that in unselected bulls heterozygous for the 1/29 translocation, the nonreturn rates at 28 and 56 d were 5 and 7% lower compared with the mean for the AI station.

Linares et al. (17) provided information on embryos sired by bulls heterozygous for the 1/29 translocation to determine if their deviation from the expected stage of development could be of significance and be used to monitor early embryonic death. It was shown that the frequency of degenerated embryos was significantly higher when the sires were heterozygous for the 1/29 translocation.

The aim of the present study was to examine the effect of using semen from a bull known to be heterozygous for the 1/29 translocation in an IVF program and to assess its affect in terms of the subsequent developmental competence of the oocytes following fertilization.

MATERIALS AND METHODS

Ovaries from post-puberal, nonstimulated heifers and cows were collected at a local abattoir after slaughter and were transported to the laboratory in sterile saline held at 35 to 37°C. On return, ovaries were washed in fresh saline and immediately processed. Primary oocytes were recovered from vesicular follicles by aspiration using an 18-gauge needle and a 5 ml syringe.

The medium used for oocyte in vitro maturation (IVM) was Tissue Culture Medium 199 with Earle's salts and 25mM Hepes (TCM 199, Sigma Chemical Co., St Louis, MO, cat. # M-7528) supplemented with L-Glutamine (200mM, Sigma, cat. # G-7513), 20% (v/v) estrous cow serum (ECS; heat-treated at 56°C for 30 min), and gentamycin (25 µg/ml, Gibco, Life Technologies, Inc., Gaithersburg, MD). The pH was adjusted to 7.4. All media were sterilized by filtration through a 0.22 µm Millipore filter and equilibrated for at least 2 h prior to use. All cultures took place in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 39°C.

For IVM, oocytes were selected on the basis of compact cumulus and uniform cytoplasm and cultured statically for 24 h in groups of 50 oocytes per 2 ml of maturation medium in 35 mm petri dishes. Additional granulosa cells obtained by centrifugation and resuspension of cells after oocyte removal from follicular aspirates were added to the maturation dishes at a concentration of 3 to 5 x 10^6 cells/ml.

Following maturation, oocytes were denuded of their surrounding expanded cumulus cells by shaking them for several minutes in a 3% solution of tri-sodium citrate. Before transfer to the fertilization droplets they were washed by transfer through 3 successive dishes of washing medium (modified hepes-buffered Tyrode's solution, pH 7.4).

Spermatozoa from 2 Norwegian Red Bulls was used, with 1 bull carrying the 1/29 translocation (Storm Kvakkestad 838, nonreturn rate 59.2% based on 1082 inseminations; semen frozen 071070) and the other (F. Torland 3939, nonreturn rate 70.6% based on 1156 inseminations; semen frozen 140389) serving as the control. A motile sample of spermatozoa for IVF was obtained by swim-up separation. Briefly, approximately 0.25 ml of frozen-thawed semen was layered under 1 ml of capacitation medium (modified Ca^{++}-free Tyrode's; pH 7.4) in conical tubes. Following incubation for 1 h, the uppermost 0.5 to 0.8 ml were removed and pooled in a conical centrifuge tube. This sample was then washed twice by centrifugation at 500g for 7 to 10 min. The resulting pellet was diluted with an equal volume of capacitation medium containing 200 µg heparin/ml (Sigma, cat. # H-3125). After incubation for 15 min the suspension was further diluted with capacitation medium to obtain the desired concentration of spermatozoa.

Fertilization was carried out in 50 µl droplets of modified Tyrode's medium (without glucose; pH 7.8) under mineral oil (Sigma, cat. # M-3516). The sperm motility stimumlating mixture PHE (containing penicillamine, hypotaurine and epinephrine) was added at the time of insemination (16). For IVF, 5 µl of the final sperm suspension were added to each droplet containing 5 oocytes to give a final concentration of approximately 1.5 x 10^6 sperm/mL

At 48 h post insemination cleaved oocytes (2 to 8 cell stage) were transferred to a previously prepared granulosa cell monolayer for a further 5 to 7 d. A representative number of blastocysts was fixed (acetic acid:methanol, 1:3) and stained (1% aceto-orcein) to assess total cell number 8 d post insemination. The results were based on cleavage rate (number of oocytes that had cleaved to the 2-cell stage or beyond at 48 h post insemination/number inseminated), blastocyst rate (number of blastocysts/number of cleaved oocytes transferred to monolayer), and blastocyst cell number.

Data were analyzed by Chi-square analysis and Student's t-test where appropriate.

RESULTS

The results of the present study are presented in Table 1. There was a significant difference between the control and carrier bull in the cleavage rate ($P<0.005$) and the blastocyst rate ($P<0.005$). However, there was no significant difference between the bulls in the number of cells in the stained blastocysts.

DISCUSSION

Reduced fertility in heterozygotes for the 1/29 translocation is thought to be due to the death of embryos with unbalanced karyotypes resulting from nondisjunction of the chromosomes comprising the trivalent at the first meiotic division (5, 22).

Gustavsson (8) reported that in the meiosis of the heterozygous carrier, the translocation and its homologues build up a (hetero)trivalent, and segregation proceeds in a fairly regular way with the production of chromosomally balanced embryos. At that time, while direct evidence for the occurrence of nondisjunctional segregation of the heterotrivalent at the first meiotic division in the form of unbalanced gametes or embryos was lacking, there was convincing indirect evidence for the occurrence of cytogenetically unbalanced zygotes which resulted in reduced fertility in the heterozygous carriers. That chromosomally unbalanced spermatozoa are produced by carrier bulls which are capable of fertilization, and that the resulting zygote has the ability to continue its development to at least the blastocyst stage is now clear from several reports in the literature (3, 20). The work of King et al. (13) provided the first direct evidence for the occurrence of chromosomally unbalanced embryos sired by bulls carrying the 1/29 translocation, suggesting nondisjunction and the formation of hyper-haploid gametes with fertilization ability.

Table 1. Use of semen from a bull with the 1/29 translocation on the outcome of in vitro fertilization and in vitro culture.

Bull	Nonreturn rate[a]	Cleavage rate %	Blastocyst rate %	Cell no.[b] (n)
838	59.2% (1082)	59.8% (382/639)[c]	12.0% (46/382)[c]	93.4±37.5 (15)
3939	70.6% (1156)	71.1% (323/454)[d]	20.0% (66/323)[d]	93.0±22.6 (17)

[a]Nonreturn rate at 60 to 90 days corrected for double insemination.
[b]Mean ± standard deviation.
[cd]Values in the same column with different superscripts differ significantly (P<0.005).
n: Number of blastocysts examined.
Bull 838 heterozygous for 1/29 translocation.
Bull 3939 control animal.
Mean of six replicates

Subsequently, King et al. (14) presented information on the karyotypes, total cell numbers and mitotic indices of embryos sired by normal and by bulls heterozygous for the 1/29 translocation. No chromosomal abnormalities were identified in the group of embryos with normal sires and dams. Of the embryos sired by heterozygous bulls that could be karyotyped, 55.3% were normal, with 39.5% being 1/29 heterozygous. The mitotic index was slightly lower in the group of Day-7 embryos with 1/29 sires than those with normal sires.

Linares et al. (17) reported a significantly higher incidence of morphologically abnormal embryos (degenerated and in the process of degeneration) sired by bulls heterozygous for the 1/29 translocation. Only 50 and 25% morphologically normal embryos were collected from naturally ovulating and superovulated donors sired by carrier bulls, respectively; whereas for embryos sired by normal bulls, the figures were 71 and 92%, respectively. As pointed out by these authors, it is not clear when embryos sired by carrier bulls die. The same authors suggest that some of the embryos probably begin to degenerate before blastocyst formation due to a lack of synchronization of cell division prior to the late morula stage, while others may be eliminated at stages beyond implantation, since daughters of the carrier bulls had higher return rates at 56 d (5, 22).

Dain and Dott (2) carried out cytogenetic studies of 1/29 translocation carrying cows and their embryos. The examination of 2 infertile heifers showed that both animals produced gametes which underwent fertilization and cell division during the first 7 d post fertilization. The same authors pointed out 1) that the translocation can be compatable with high fertility in cows, suggesting that the rearrangement caused by the translocation can sometimes be accomodated but in other cases upsets meiosis, perhaps by altering the fine structure and hence the timing of disjunction, and 2) that there is a difference in survival times of aborted embryos which have 1 translocation carrying parent, with gestation time varying from the first few days preceding implantation to a period of several weeks after it.

The results of the present study clearly indicate that spermatozoa produced by a carrier bull are capable of fertilizing in vitro-matured oocytes and that the resulting zygotes are capable of developing to at least the blastocyst stage following culture in vitro. While the number of blastocysts produced by the carrier bull was significantly lower than that produced by the control bull, the blastocyst quality, based on cell number at 8 d post insemination, was similar. It should be pointed out that, although not specifically assessed, such embryos were apparently capable of hatching from the zona pellucida with a similar frequency to controls.

In the present study there was a close relationship between the results observed in vitro and the field fertility records based on nonreturn rate. Such observations are consistent with previous reports (12, 18, 26, 27). It is now well known that, even in the absence of known genetic factors, considerable variation exists between bulls in their ability to fertilize oocytes in vitro (12, 26) and that embryo development may differ among bulls even when fertilization rates are similar. Indeed, the results of Otoi et al. (19) showed that embryo development rates may differ even among different ejaculates from the same bull. Bearing this in mind, and in the absence of supporting karyotyping data it can be concluded that the observed difference between the two bulls may be due to the presence of the 1/29 translocation, but not necessarily.

To the authors' knowledge, this is the first report in the literature concerning the use of semen from a bull known to be heterozygous for the 1/29 translocation in an IVF program. A paper by Heuwieser et al. (11) described the use of semen from a Brown Swiss bull with a history of 1/16 tandem translocation with trisomy 16 (15). A nonreturn rate of 42% was observed based on 140 first inseminations. The fertilization rate achieved following IVF was only 1%, although this was increased to 21% following the microinjection of spermatozoa.

REFERENCES

1. Amrud J, Nes N. Chromosome investigation in animals with congenital defects. X Nordiska Veterinarmotet 1966; II: 585-587.
2. Dain AR, Dott HM. Cytogenetic studies of 1-29 translocation carrying cows and their embryos. Theriogenology 1985; 23: 641-653.
3. Dyrendahl I, Gustavsson I. Sexual functions, semen characteristics and fertility in bulls carrying the 1/29 translocation. Hereditas 1979; 90: 281-289.
4. Gustavsson I. Chromosome abnormality in cattle. Nature 1966; 211: 865-866.
5. Gustavsson I. Cytogenetics, distribution and phenotypic effects of a translocation in Swedish cattle. Hereditas 1969; 63/ 68-69.
6. Gustavsson I. Culling rates in daughters of sires with a translocation of centric fusion type. Hereditas 1971; 67: 65-74.
7. Gustavsson I. Chromosomes of repeat-breeder heifers. Hereditas 1971; 68: 331-332.
8. Gustavsson I. Distribution and effects of the 1/29 translocation in cattle. J Dairy Sci 1979; 62: 825-835.
9. Gustavsson I, Rockborn G. Chromosome abnormalities in three cases of lymphatic leukemia in cattle. Nature 1964; 203: 990 abstr.
10. Herschler MS, Fechheimer NS. Centric fusion of chromosomes in a set of bovine triplets. Cytogenetics 1966; 5: 307-312.
11. Heuwieser W, Yang X, Jiang, S , Foote RH. A comparison between in vitro fertilization and microinjection of immobilized spermatozoa from bulls producing spermatozoa with defects. Mol Reprod Dev 1992; 33: 489-491.
12. Hillery FL, Parrish JJ, First, NL. Bull specific effect on fertilization and embryonic development in vitro. Theriogenology 1990; 33: 249 abstr.
13. King WA, Linares T, Gustavsson I, Bane A. Presumptive translocation type trisomy in embryos sired by bulls heterozygous for the 1/29 translocation. Hereditas 1980; 92: 167-169.
14. King WA, Linares T, Gustavsson I. Cytogenetics of preimplantation embryos sired by bulls heterozygous for the 1/29 translocation. Hereditas 1981; 94: 219-224.
15. Kovacs A, Foote, RH, Lein DH. 1;16 tandem translocation with trisomy 16 in a Brown Swiss bull. Vet Rec 1990; 127: 205
16. Leibfried ML, Bavister BD. Effects of epinephrine and hypotaurine on in vitro fertilization in the Golden Hamster. J Reprod Fertil 1982; 66: 87-93.

17. Linares T, King WA, Gustavsson I. Morphological appearance of bovine embryos sired by bulls heterozygous for the 1/29 translocation. Proceedings of the 4th European Colloquim on Cytogenetics of Domestic Animals. 1980; 188-193.
18. Marquant-Le Guienne B, Humblot P, Guillon N, Gerard O, Thibier M. In vitro fertilization as a tool to evaluate fertility in the bovine. Proc 12th Int Cong Anim Reprod 1992; 2: 662-664.
19. Otoi T, Tachikawa S, Kondo S, Suzuki T. Effects of different lots of semen from the same bull on in vitro development of bovine oocytes fertilized in vitro. Theriogenology 1993; 39: 713-718.
20. Popescu CP. Cytogenetic study on embryos sired by a bull carrier of 1/29 translocation. proceedings of the 4th European Colloquim on Cytogenetics of Domestic Animals 1980; 182-187.
21. Popescu CP, Pech A. Cattle 1/29 translocation in the world (1964-1990): a review. Ann Zootech 1991; 40: 271-305.
22. Refsdal AO. Low fertility in daughters of bulls with 1/29 translocation. Acta Vet Scand 1976; 17: 190-195.
23. Reiger R, Michaelis A, Green MM. A Glossary of Genetics and Cytogenetics. Springer-Verlag, Berlin, 1968.
24. Rieck GW, Hohn H, Herzog A. Familiares Vorkommen der zentromeren Chromosomenfusion beim Rind. Zuchthyg 1968; 3: 177-182.
25. Rugiati S, Fedrigo M. Alterazione cromosomomiche riscontrata in un toro acondroplasico di razza romagnola. Acta Biol Med 1967; 38: 522.
26. Shi DS, Lu KH, Gordon I. Effects of bulls on fertilization of bovine oocytes and their subsequent development in vitro. Theriogenology 1990; 33: 324 abstr.
27. Shi DS, Lu KH, Gordon I, Polge C. Variation in cleavage and embryonic development of bovine oocytes in vitro fertilized with different bull ejaculates. Theriogenology 1991; 35: 271 abstr.

MEDIA PREPARATION

FOR

BOVINE

IN VITRO EMBRYO PRODUCTION

University College Dublin

Enero 2005

Slaughter house PBS

Dissolve 5 PBS tablets (Oxoid BR0014G) in 500 ml of de-ionized distilled water and steam sterilized at 121 oC for 15 min

Laboratory PBS

A

		mg/litre	mg/2litres
NaCl	Merck 6404	8000	16000
KCl	prolabo 26764298	200	400
KH_2PO_4	Prolabo 26926298	200	400
$Na_2HPO_4\ 2H_2O$		1430	2860

Dissolve in 500/or 1000 ml of mQ

B

		mg/litre	mg/2litres
$CaCl_2\ 2H_2O$	Merck 2382	140	280
$MgCl_2\ 6H_2O$	Merck 5833	200	400

Dissolve completely to avoid precipitation in 100/or 200ml of mQ

Add to **A & B**

		mg/litre	mg/litres
D-glucose		1000	2000
Pyruvate		36	72
Gentamycine		5ml	10ml

Add sterile (Sigma water W1503) water up to 1or 2 liters

Check Osmolarity = should be around 280 mOsm (We do not adjust this osmolarity)

Then Add 0.5 gr BSA/litre (dissolve well) – Sigma fraction V, A9647.

PH = should be 7.2 - 7.4
Filter through 0.22 µm filter
Store at 4 ^{0}C for maximum one month.

PERCOLL

Stocks Minerals

1. KCl 1 M	: 0.745 gr/10ml	prolabo 26764298
2. NaH_2PO_4 0.1 M	: 0.138 gr/10ml	Merck 6346 H2O
3. $CaCl_2$ 1 M	: 1.47 gr/10ml	Merck 2382 2H2O
4. $MgCl_2$ 0.1 M	: 0.203 gr/10ml	Merck 5833 6H2O

NB: Stock 3 and 4 must be stored at 4 oC for weekly percol preparation

Stock 10X

4.675 gr NaCl	Merck 6404
2.38 gr Hepes	Sigma H 3375

3.1 ml KCl 1M
2.9 ml NaH_2PO_4 0.1 M

Add sterile (SigmaWater) water up to 60 ml

Adjust PH = 7.4 (NaOH 4N)

Add sterile (Sigma Water) water up to 100 ml

Filter and aliquot in 10 ml amounts and freeze at –20 °C

(The stock 10x that is in use can be stored at 4 °C)

Percoll 90

(This Recipe will make 25mls)

52 mg Bicarbonate	Sigma S 5761
50 µl $CaCl_2$ 1 M	
100 µl $MgCl_2$ 0.1 M	
92 µl Lactate syrup	Sigma L 4263
2.5 ml Stock 10X	
Dissolve well	
Add 22.5 ml Percoll	Pharmacia

Store for 1 week maximumat 4 oC

TYRODE 'S MEDIUM (Basic medium for Fert, STL and STL-BSA)

	Reference	mM	mg/500ml	mg/litre
NaCL	Merck 6404	100	3333	6666
KCl	Prolabo 26764298	3.1	120	240
$NaH_2PO_4 * H_2O$	Merck 6346	0.3	20	41
$CaCl_2 * 2H_2O$	Merck 2382	2.1	150	300
$MgCl_2 * 6H_2O$	Merck 5833	0.4	50	100
Phenol Red	Merck 7241		5	10

Dissolve well (sigma water) and top up to relevant volume (500ml or 1Lt) and check osmolarity. Should be very close to 230 otherwise remakes the media. (We do not adjust this osmolarity)

To make **Fertilization medium,** Washing medium (**STL** and **STL-BSA**)
Prepare the following beakers:

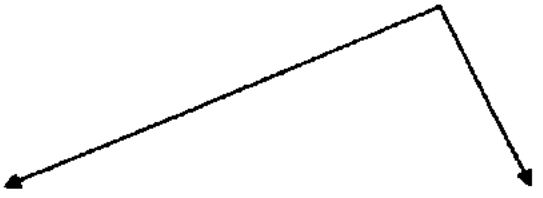
1. 650 mg Bicarbonate	Sigma S 5761
34 mg Pyruvate	Sigma P 3662
2. 600 mg BSA (Fatty acid free)	Sigma A 6003
3. 480 mg Hepes	Sigma H 3375
4. 840 mg BSA	Sigma A 9647

Take 300 ml of Tyrode's Medium and add:

- Contents of Beaker 1
- 0.92 ml lactate syrup — Sigma L 4263
- 1.48 ml Gentamycin — Gibco 043-05710 D

300 ml

Divide: 100 ml and 200 ml
Add: Beaker 2 Beaker 3
(Fertilisation medium)



140 ml 60 ml

Add: Beaker 4

(STL Medium Without BSA)

(STL Medium)

Fert Media:
Filter an aliquot in 10 ml exactly and store at 4 oC
STL
Filter and aliquot in tubes (aprox 12 ml each) and store at 4 oC
STL-BSA
Filter and aliquot in 4 tubes (aprox 12 ml each) and store at 4 oC

Above media must be used within 2 weeks

Heparin Preparation

Weigh "X" amount in mg of Heparin and equal volume in mls of Sigma water. This concentration is 1mg/ml
Filter and aliquote in 110ul amounts and store at –20 oC

eg: Weigh 10mg of Heparin and add 10ml of Sigma water

Note: 100ul of Heparin is added to the 10 mls of Fert prior to use giving a final concentration of 10ug/ml

Composition of Synthetic Oviduct Fluid (SOF)

		Reference	MW	Molarity (mM)	mg/500ml
1	NaCl	Merck 6404	58.44	107.70	3147
2	KCl	Prolabo 26 764 298	74.55	7.16	267
3	KH_2PO_4	Prolabo 26 926 298	136.09	1.19	81
4	$CaCl_2*2H_2O$	Merck 2382	147.02	1.71	126
5	$MgCl_2*6H_2O$	Merck 5833	203.3	0.49	49
6	$NaHCO_3$	Sigma S-5761	84.01	25.07	1053
7	Na lactate	Sigma L-4263	112.1	3.30	141µl
8	Na Pyruvate	Sigma P-3662	110.0	0.30	17
9	Phenol Red	Merck 7241	354.38	1.3µg/ml	0.65
	Glutamine 200mM	Gibco 043-05030H		1.0	2.5ml
	aa essentials (BME)	Sigma B-6466			10ml
	aa non essentials (MEM)	Sigma M-7145			5ml
	BSA	Sigma A-9647		3.0g/ml	1500
	Gentamycin	Sigma G-1272			2.5ml

A) Dissolve the salts (1,2,3, 6, 8 &9) in ~ 300ml of Sigma Water

B) Dissolve CaCl2 (3) and MgCl2 (4) in 100ml.

C) Mix both solutions when dissolved completely and add :

Na lactate, Glutamine, Amino Acids, Gentamycin.

D) Adjust the volume to 500 mls and check Osmolarity (270-280: normally this is within range, if not remake the media).

E) Add the BSA and measure the pH (7.2 – 7.4: if slightly out of range adjustment can be made with 1 N NaOH)

Filter through 0.22 µm filter .

Aliquot in 15 ml volumes and store at 4 ^{0}C for maximum two weeks.

Composition of Holm Synthetic Oviduct Fluid

Stock A: 10 X solution Store 4 oC for 1 month

		Reference	MW (g/mol)	gr/100ml
1	NaCl	Merck 6404	58.44	6.290
2	KCl	Prolabo 26 764 298	74.55	0.534
3	KH_2PO_4	Prolabo 26 926 298	136.09	0.162
4	$MgSO_4*7H_2O$		120.4	0.182
5	Na lactate (ml) Add last	Sigma L-4263	112.1	0.600
6	Sigma Water (ml)			99.4

Filter sterilized 0.22
NB: This stock A is used for SOF B (Sheep) preparation

Stock B: 10 x Bicarbonate-phenol red solution (Store 1 month at 4 oC)

		Reference	MW	gr/100ml
1	$NaHCO_3$	Sigma S-5761	84.01	2.100
2	Phenol Red	Merck 7241	354.38	0.010

Filter 0.22

Stock C: 100x Pyruvate solution (Store 1 week at 4 oC)

		Reference	MW	gr/10ml
1	Na-Pyruvate			0.0800
2	Sigma Water (ml)			10

Filter 0.22

Stock D: 100x $CaCl_2$ solution (Store 1 month at 4 oC)

		Reference	MW	gr/50ml
1	$CaCl_2 * 2H_2O$			1.3100
2	Sigma Water (ml)			50

Filter 0.22
NB: This stock D is used for SOF B (Sheep) preparation

SOF-IVC Medium Preparation

	Reference	MW	gr/100ml
Sigma Water (ml)	W 1503		78
Tri-Sodium Citrate			0.0100
Myo-inositol			0.500
SOF-Stock A (ml)			10
SOF-Stock B (ml)			10
SOF-Stock C (ml)			1
SOF-Stock D (ml)			1
BME (ml)			3
MEM (ml)			1
L-Glutamine (ml)			100 ul
Gentomycin (ml)			500 ul

Check osmolarity: should be 275-285 mOsm (if not in range remake media)
Filter 0.22. Aliquot in 9.5 ml volumes and store in 4 oC for 1 week

Note: Prior to use add 5% of FCS

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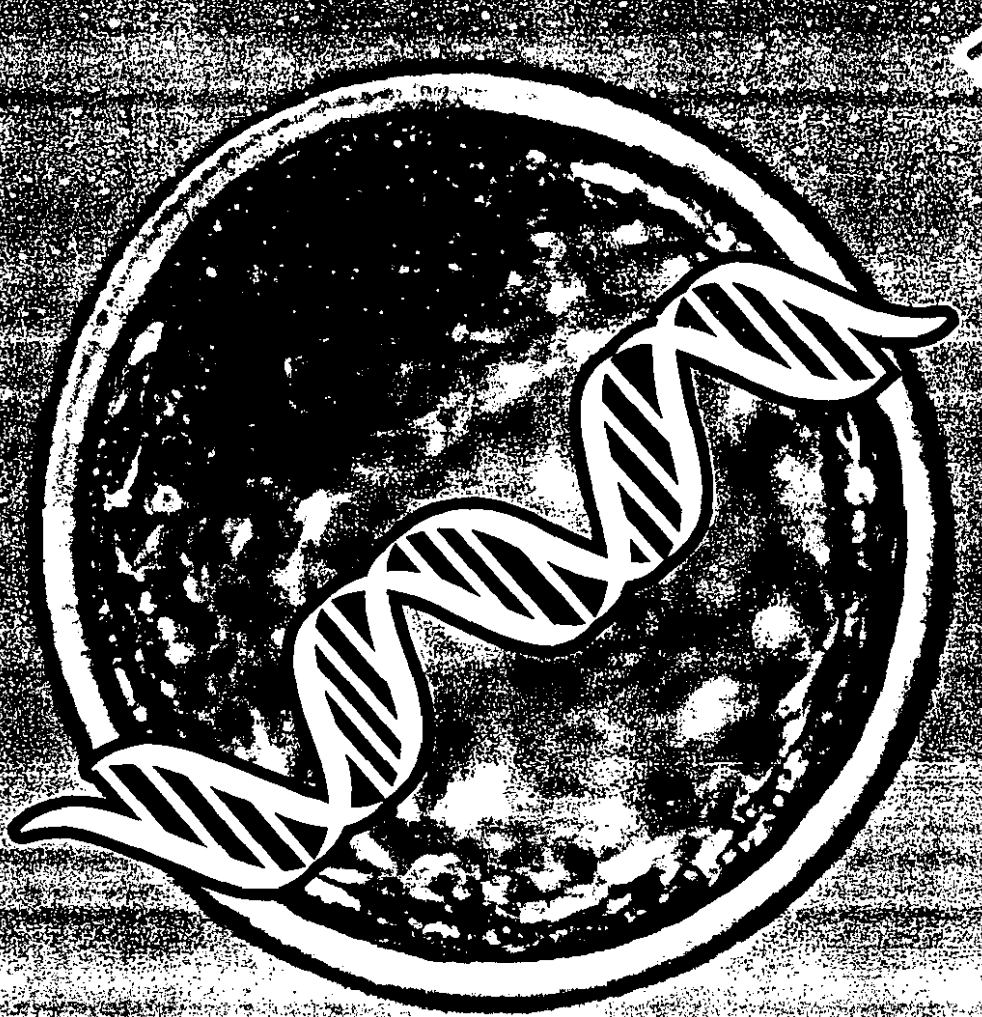

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MARCO BERLAND

PROCEEDINGS OF THE ANNUAL CONFERENCE OF THE INTERNATIONAL EMBRYO TRANSFER SOCIETY

Copenhagen, Denmark

8–12 January 2005

Main Theme: Progress in Understanding Mammalian Oocyte and Embryo Development

Edited on behalf of the Society by

Program Chair
Heiner Niemann
Institut Für Tierzucht
Neustadt, Germany

Program Co-Chair
Giovanna Lazzari
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Cremona, Italy

International Embryo Transfer Society
Jennifer Gavel, Executive Secretary
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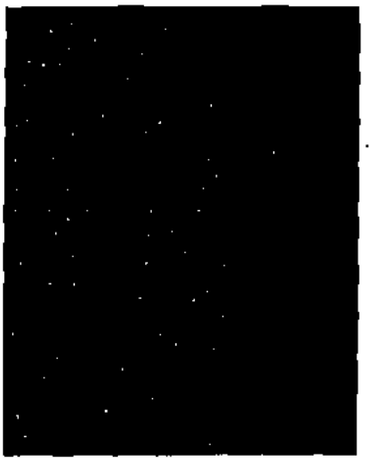
Program Book

The 31st Annual Conference of The International Embryo Transfer Society

The Falconer Center
and
The Royal Veterinary and Agricultural University
Copenhagen, Denmark
January 9-11, 2005

Co-Chairs of the Scientific Program:
Heiner Niemann and Giovanna Lazzari

M. BERLAND



IETS Satellite Symposium

Agricultural and societal implications of contemporary embryo-technologies in farm animals

Royal Veterinary and Agricultural University

Copenhagen, Denmark

Wednesday, 12 January 2005

Symposium sponsors

OECD

BD Biosciences

The Abildgaard Foundation

DakoCytomation

KVL

A welcome from the symposium organizers

It is great pleasure to welcome you to this IETS satellite symposium. We hope that the scientifically tightly packed IETS Annual Conference left some energy and enthusiasm for this post-conference. There are some very interesting presentations and we hope that they will give rise to plenty of discussions.

For this symposium we have chosen a subject that should attract the attention towards the future. At the 1997 IETS Annual Conference in Nice, France, Theo Kruip and Nanke den Daas presented a review paper entitled "In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring". This was an eyeopener for many of us and gave clear proof for the adverse effects of IVF and SCNT procedures on the course of pregnancy, parturition and offspring. In 2003, Theo Kruip passed away and we have asked Janneke to dedicate her lecture to Theo.

Over the last decade, a lot of work has been done, both in the field of embryo technology and in semen research. It has therefore been decided to let the morning presentations of this symposium address these two main subjects in parallel sessions. For the afternoon, we all then gather to hear lecturers addressing the future from various points of view.

We wish to express our sincere thanks to the distinguished colleagues and friends who have agreed to give a presentation. You will find a summary of their presentations in the small abstract book, in which you are now reading. It should be added that full size papers of their presentations will later appear in a special issue of *Theriogenology*.

Finally, we are delighted to thank the sponsors of this symposium, namely OECD, the Abildgaard Foundation and Royal Veterinary and Agricultural University (KVL). We are particular indebted to OECD, where the Management Committee of the Co-operative Research Programme: Biological Resource and Management of Sustainable Agriculture Systems chaired by Dr. Allan King, decided to support the travel of most of the speakers. The Abildgaard Foundation enabled us to invite the remaining speakers and has also contributed to the lunch and coffee breaks. Finally did KVL kindly provide housing for the symposium.

We wish you all a good and informative day!

Frederiksberg, 12 January 2005

Torben Greve and Henrik Callesen

Program

08.15-08.45: Registration (Marble Hall of KVL)

Parallel Session 1: Embryo technologies and animal health – an international update (Auditorium 3.01)
09.00-09.15: Torben Greve, Denmark, Introduction and Chair of Session
09.15-09.45: Keith J. Betteridge, Canada: "Domestic animal embryo-technologies: achievements and perspectives"
09.45-10.30: **Theo Kruip memorial lecture** given by Janneke van Wagtendonk-de Leeuw, New Zealand: "OPU and IVP after use in several generations, a 2005 status"

10.30-11.00: Break (Marble Hall)

11.00-11.45: Tom McEvoy, Scotland: "Consequences for the animal following OPU, IVP and somatic cell nuclear transfer"
11.45-12.15: Torben Greve, Denmark: Discussion and concluding remarks

Parallel Session 2: Semen technologies and animal health – an international update (Auditorium 3.13)
09.00-09.15: Preben Christensen, Denmark, Introduction and Chair of Session
09.15-09.45: Duane L. Garner, USA: "Flow cytometric sexing of mammalian sperm"
09.45-10.30: Bart M. Gadella, The Netherlands: "Detection of damage to DNA in mammalian sperm"

10.30-11.00: Break (Marble Hall)

11.00-11.45: Donald P. Evenson, USA: "Clinical aspects of detection of DNA fragmentation in male infertility and comparison with other techniques"
11.45-12.15: Discussion and concluding remarks (Preben Christensen, Denmark)

12.15-13.30: Lunch (Marble Hall)

Session 3: The next 10 years with semen and embryo technologies (Auditorium 3.01)
13.30-13.45: Allan King, Canada, Introduction and Chair of Session
13.45-14.15: Ian Wilmut, UK: "Agricultural applications in research and industry"
14.15-14.45: Peter Sandøe, Denmark: "After Dolly – ethical limits to the use of biotechnology on farm animals"

14.45-15.15: Break (Marble Hall)

Concluding part (chaired by Henrik Callesen, Auditorium 3.01)
15.15-16.00: Panel Discussion (King, Wilmut, Sandøe)

DOMESTIC ANIMAL EMBRYO TECHNOLOGIES: ACHIEVEMENTS AND PERSPECTIVES

Keith J. Betteridge
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Embryo technology (or embryo transfer, ET) was as fashionable a term 30 years ago as it is today, but the procedures it conjures now were unimaginable then. Despite the enormous changes, however, the embryo itself, its survival of transfer, and the quality of the animal it produces still measure the success of any embryo manipulation. Therefore, progress in embryo technology can be gauged in terms of how well we can make and use embryos, and how much they have taught us about the maintenance of pregnancy and reproduction in general. The extraordinary progress and diversification in what was initially a narrow field should also lead us to consider how well, or poorly, our education prepared us for coping with rapid change, and how we might improve the preparation of our successors. Thus the twin themes of progress made and lessons learned will be explored as a means of giving perspective the work that we do.

I shall argue that progress has come through collaboration and the exchange of information. The collegiality of veterinarians, animal scientists and biologists in the International Embryo Transfer Society is exemplary and has paid important dividends, notably in the elaboration of internationally accepted procedures for moving embryos around the world. Recent reviews of the history and current status of ET will be mentioned with emphasis on the "three generations" of ET in cattle – the first with embryos derived from donors (in vivo), the second with embryos produced in vitro, and the third including further in-vitro techniques, notably somatic cell nuclear transfer and transgenesis. However, rather than attempting to review this immense field even briefly, I shall consider four generalities that have emerged during the development of ET and which are, I think, of enduring relevance.

The first generality is that the failures that have accompanied the development of ET – especially its in-vitro aspects – are as instructive as the successes. Investigation of early embryonic loss and the "Large Offspring Syndrome", for example, have focused attention on the epigenetic effects of the environment from the earliest stages of development.

The second generality is that ET research makes us rethink some established dogmas in reproduction. Thus, early notions that "genotype is everything" (and the recipient relatively unimportant), which have underpinned the application of ET, clearly need to be tempered as our knowledge of epigenetics extends.

The third generality is that initially complex techniques become simplified as they are put to use in the field. ET itself, embryo manipulation and cryopreservation are examples and cloning may follow. The simpler the technique, the wider its applicability, and the better the prospects for using it to benefit animal production in less advanced countries.

My final generality is an opinion: that a veterinary education is an excellent starting point for those keen to advance embryo technology. For a research career, that education will have to be extended of course, but a broad appreciation of the whole animal, as well as its gametes and embryos, is a decided advantage. As scientists or practitioners working with embryos, our knowledge of animals and their welfare will play an increasingly important role in influencing public perception of embryo technology, potentially a major impediment to its use.

OVUM PICK UP AND IN VITRO PRODUCTION IN THE BOVINE AFTER USE IN SEVERAL GENERATIONS: A 2005 STATUS

A.M. van Wagtendonk-de Leeuw
Livestock Improvement Corporation, Private bag 3016, Hamilton, New Zealand

The first In Vitro Produced (IVP) calf was born in 1982 and the non-surgical Ovum Pick Up (OPU) technique for the bovine was adapted from the human in 1987. Since then, considerable research has been aimed at improving both technologies in the bovine. Major breakthroughs in efficiency and/or efficacy in OPU have been through the use of disposable needles, increased frequency of collection and hormonal pre-stimulation prior to OPU. In IVP breakthroughs have been the use of immature rather than mature oocytes, overcoming the 8-16 cell block through low oxygen environments for in vitro culture, the removal of serum from culture media and the re-discovery of the Synthetic Oviductal Fluid medium (originating from 1972) to decrease the symptoms of the Large Offspring Syndrome (LOS).

Both OPU and IVP can now be seen as mature technologies. It can be estimated that more than 200,000 IVP calves have been born world wide since 1996, and when the two technologies are combined they are capable of producing over 50 calves per donor cow per year, however with a large variation between donors. In contrast to MOET, OPU/IVP will produce offspring from nearly every donor cow, will generate more offspring per time unit, is less dependent on the reproductive status of the donor (offspring from immature calves and pregnant cows can be produced) and several bulls rather than 1 can be used to fertilize oocytes from one OPU session. It requires, however, a more demanding laboratory set-up compared to MOET.

Not many new breakthroughs are expected for OPU. For IVP however, automation and miniaturization as well as a greater understanding of the embryo through the application of genomic technologies such as micro-arrays, may provide an environment that is more in-vivo like than traditional micro drop/well systems. This improved environment should result in higher embryo development rates as well as improved quality and welfare of subsequent offspring in terms of birth weight, congenital abnormalities and viability.

The application of OPU/IVP has progressed from treating infertile MOET cows in commercial situations to influencing the design of breeding schemes. The main advantage of OPU/IVP over MOET is the much more consistent production of offspring, in spite of the variation already mentioned, thus greatly enhancing the planning and efficacy of breeding schemes. A higher increase in selection intensity on the female side by producing multiple offspring per donor cow, as well as the increase in selection accuracy by generating full- and half sib families has resulted in higher genetic gain in breeding schemes using OPU/IVP compared to MOET. Application of OPU/IVP on juvenile animals (2-3 months of age) compared to mature animals (over 14 months of age) reduces the generation interval, but is counteracted by a decrease in selection accuracy since the information on parent performance is not yet available at the time of selection. Worldwide, the numbers of bovine OPU/IVP sessions are currently decreasing (with significant differences between countries), because of the high cost of the technology and the reduced expectations of an improvement in results with an accompanying reduction in costs.

With the bovine genome being rapidly sequenced and bovine genes for traits of economic interest becoming available in the next years, OPU/IVP will prove invaluable in rapidly

multiplying rare genes or Quantitative Trait Loci (QTL) of high value. Bull breeding and the subsequent dissemination of the genes through AI (in case of genes coding for animal productivity traits such as health and fertility) and more importantly through herd population (in the case of genes for milk composition traits) will be more efficient and effective through OPU/IVP compared to MOET. Juvenile OPU/IVP will further accelerate gene dissemination.

In due time, it is anticipated that Marker Assisted Selection or Gene Assisted Selection (MAS/GAS) schemes in combination with sib testing may (partially) replace conventional progeny testing schemes and provide opportunities for customizing animals to different markets. MAS/GAS is already applied at several places in the world. It is recognized that OPU, and particularly IVP, provide the base for more advanced technologies such as cloning and transgenics, which are believed to have limited scope due to animal welfare and public perception issues. This is likely to maintain, until the first convincing examples of positive uses are presented, possibly first within areas outside of the farming industry but within the medical or pharmaceutical industry.

This paper is dedicated to celebrate and recognize the significant contributions made by Theo Kruip to the wide area of bovine OPU and IVP.

EMBRYOTECHNOLOGIES AND ANIMAL HEALTH - CONSEQUENCES FOR THE ANIMAL FOLLOWING OVUM PICK-UP (OPU), *IN VITRO* PRODUCTION (IVP) AND SOMATIC CELL NUCLEAR TRANSFER

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Mammalian reproductive technologies that aim either to complement or to transcend conventional livestock breeding options have contributed to some of the most remarkable achievements in the field of reproductive biology in recent decades. In so doing they have extended our horizons in two distinct dimensions, the first concerning what it is technically possible to achieve and the second relating to the time-frame within which an individual's life-long developmental capability is initially established and ultimately realized or under-mined. Our impressions of the benefits and values, or otherwise, of technologies such as *in vitro* embryo production and nuclear transfer are rightly influenced by the extent to which they influence the health of animals either subjected to or derived from them. Here we consider some of the health implications of technologies applied to farm livestock and centred on the oocyte or embryo.

Ovum pick-up (OPU) technologies have been used to harvest oocytes repeatedly from either gonadotrophin-stimulated or unstimulated donor animals, the latter instance being associated for the most part with collection protocols that, when applied once-weekly in cattle, capitalise on the wave-like emergence and growth of their ovarian follicles and in so doing usually modify both behavioural and endocrine patterns associated with the animal's natural oestrous cycle. Less frequent OPU tends to include follicle ablation or administration of exogenous gonadotrophins or both. Whether such protocols have significant or lasting effects on the animals concerned is a question that has not been fully answered. Some reports have identified changes brought about in the ovary – mainly scarring and textural modifications – but these have tended not to compromise subsequent gonadal function in the donor. A study at SAC investigated the legacy of repeated epidural anaesthesia in terms of injection site injuries and indicated that, provided protocols are properly applied, in particular to maintain asepsis, the animal's health is not compromised significantly.

The health implications for animals generated via *in vitro* production of intact embryos seem more varied in the case of ruminant species than, for example, in pigs. A case in point is the 'Large Offspring Syndrome', an enigmatic and multi-variate phenomenon of unpredictable frequency. The syndrome is indicative of developmental sensitivities and aberrations that undermine the viability of young derived from IVP embryos and in circumstances of severe dystocia, that of their surrogate dams. Anomalies that contribute to losses during gestation include compromised placental competence, while perinatal deaths tend to be due to cardiovascular, pulmonary or other limitations, often but not always aggravated by foetal oversize or parturient disorders. While some therapies or interventions can rescue vulnerable offspring, there may remain inherent defects that subsequently result in ill-thrift or sudden deaths.

Somatic cell nuclear transfer-derived individuals of all farm animal species manifest many of the developmental anomalies now associated with exposure of intact ruminant livestock embryos to *in vitro* environments. Effects are sometimes more severe, however, and the incidence of failure is understandably higher in view of the additional traumas and developmental re-programming requirements associated with the technology. When nuclear transfer is used to generate transgenic livestock, further determining factors include the contribution of transgene expression *per se* to health of the offspring. If disease resistance is the goal, success should enhance animal welfare.

FLOW CYTOMETRIC SEXING OF MAMMALIAN SPERM

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This review re-examines parameters needed for optimization of flow cytometric sexing mammalian sperm and updates the current status of sperm sexing in species where this technology is currently being applied. Differences in DNA content have provided not only a method to differentiate between these sex-determining gametes, but serve as a basis for predetermining sex in mammals. Although the DNA content of all cells for each mammalian species is highly conserved, slight DNA content differences of sperm occur within species and even among cattle breeds. Most mammals produce flattened, oval-headed sperm that can be oriented within a sorter using hydrodynamic forces. An approximated sorting index using the difference in DNA content of the X- or Y-chromosome bearing sperm and the area of the flat profile of the sperm head suggests that bull and boar sperm are well suited for separation in a flow sorter. Successful sperm sexing of other species must take into account the relative susceptibilities of gametes to the stresses that occur during sexing. Sorting conditions must be optimized for each species to achieve acceptable sexing efficiency for sperm from a variety of mammalian species. In the commercial application of sperm sexing to cattle, the fertility of sex-sorted bull sperm at 2 x 10^6/dose remains at 70 to 80% of unsexed sperm at normal doses of 10 to 20 x 10^6 sperm. DNA content measurements have provided the precision necessary to identify the X- and Y-chromosome bearing sperm populations in semen from at least 23 mammals while normal-appearing offspring have been produced from sexed sperm in at least 7 species.

DETECTION OF DAMAGE IN MAMMALIAN SPERM CELLS

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A number of staining protocols have been developed to detect the membrane and organelle integrity of mammalian sperm cells.

Membrane-integrity is usually assessed after staining cells with membrane impermeable dyes. Those cells that are capable to exclude these dyes can be considered to be alive. An array of membrane-impermeable fluorescent probes with affinity for DNA is currently used for this purpose. An alternatively (or simultaneously) way to study membrane integrity is to use acylated membrane dyes. By virtue of their acyl-moiety these membrane probes they can pass the intact membrane and enter living sperm cells. Entered probes are immediately be deacylated by cytosolic esterases leaving the probe membrane impermeable. Thus living sperm cells become loaded with probe, whereas, it leaks out of cells with damaged membranes. The combination assay of SYBR-1 to color living (green fluorescent) and ethidium homodimer to color deteriorated (red fluorescent) cells simultaneously is commercial available.

Organelle-specific dyes are commonly used to detect functionality of mitochondria or the acrosome. Acrosome integrity is commonly measured with fluorescent conjugated lectins that bind to acrosome specific carbohydrate moieties. Depending on the species lectin conjugates of Pisum sativum (PSA), or of peanut (PNA) are used. Detection can be done on living sperm (no fluorescence is indicative for an intact acrosome, fluorescence is indicative for acrosome disruption or acrosome reaction) or on permeabilized sperm cells (full fluorescent acrosomes are supposed to be intact whereas acrosomes with lower, patchy or equatorial band fluorescent staining are disrupted or acrosome reacting). Mitochondria can be stained with mitotracker dyes that specifically stain these organelles. Some of the mitotracker dyes change their fluorescent properties due to changes in the potential of the inner mitochondrial membrane (IMM) and thus can be used to report depolarization of the IMM. Since only polarized IMM are able to produce ATP aerobically, these dyes enable to detect mitochondrial functionality.

Other new developments are the use of ratiometric probes like C11BODIPY fatty acids analogs used to report lipid peroxidation: The intact probe is red fluorescent but turns into green fluorescence when oxidized. This green formation is believed to indicate membrane damage. Merocyanin as well as fluorescent annexin V monitor more disordered phospholipid arrangements in the sperm plasma membrane bilayer. These changes are linked to initial membrane damage during cryostorage or to early capacitation-specific membrane alterations.

Besides the importance of functional integrity of sperm membranes and organelles for fertilization, the integrity of the paternal DNA is of crucial importance for the further development of an embryo. DNA damage can be measured at different levels and for sperm DNA principally three different approaches are currently used. Firstly, the DNA of matured sperm cells (probably the fertilization competent subpopulation) is extremely highly condensed to protamines. The condensation status of individual sperm cells can be assessed using Transmission Electron Microscopy (TEM) in which condensed nuclei appear homogeneously black in contrast to non-condensed nuclei. Using COMET discrimination can be made between fluorescently labeled DNA of normally condensed sperm nuclei (minimal migration) and more loosely packed DNA (tailing of DNA) after allowing DNA migration on an agarose gel under an electric field. The second level is to detect whether sperm DNA is double stranded (intact) or

whether single stranded DNA (damaged, for example in nicks) are formed. Acridine orange can be used to stain single stranded DNA (red fluorescent) and double stranded DNA (green fluorescent). The other method is to allow enzymatic incorporation of fluorescent nucleotide analogs into single stranded DNA areas by TUNEL: fluorescent sperm cells contain single stranded DNA. The third level to detect DNA damage is to look at nuclear/DNA fragmentation, which is (unlike the above mentioned other DNA damage levels) supposed to be specific for apoptosis. Nuclear fragments can be imaged by TEM or fluorescently after staining the sperm with a DNA probe. Furthermore, DNA fragmentation can be visualized by gelelectrophoresis howing inter-histone DNA laddering. This is an unlikely process in normal sperm cells that contain: The DNA is highly condensed but not to histones and the cells apparently lack an apoptotic machinery.

AGRICULTURAL APPLICATIONS IN RESEARCH AND INDUSTRY

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The applications that are being developed fall into two broad categories. They are for genetic modification or for multiplication of specific genotypes. New opportunities for the genetic modification of livestock will be created by the availability of information on genome sequences and the development of several novel methods for precise genetic change. Efficient gene transfer is being achieved by use of lentiviral vectors in both poultry and mammals. In addition to gene addition studies, in due time it is to be expected that these vectors will also be able to offer tissue specific expression of RNAi constructs. This will provide a comparatively cheap opportunity to reduce the production of specific proteins in particular tissues in livestock. At present nuclear transfer from cells in which precise genetic modifications have been made offers the only route to total deletion of gene function or modification of the gene product. While this has been achieved in sheep, pigs and cattle it remains a technically challenging and expensive procedure. Cloning has not yet been described in poultry. This approach is limited by the lack of cell populations that can be maintained in culture for a sufficient period to allow targeting, selection and testing to confirm that the desired change has been introduced and the inadequacies of the present cloning procedures.

Several procedures for oocyte maturation, embryo culture and embryo manipulation have the potential to increase the number of offspring available from selected mating. Procedures for maturation of full-grown oocytes and fertilisation have become more successful in mammals. Embryo culture is comparatively effective in mammals and chicken. However, progress is still limited in research to support growth and maturation of oocytes from small follicles. Evidence in the mouse and human that gametes may be derived from embryo stem cells offers the prospect of being able to use comparable methods in selection programmes once stem cells are derived from livestock. The potential value of nuclear transfer is well recognised, but much remains to be achieved to improve the efficiency of the procedure before that potential can be delivered.

In the first place it seems likely that any new procedure will be used first in research, particularly those concerned with genetic modification. In some parts of the world the consumers have made it clear that they do not support proposals to genetically modified animals. The populations of countries in which the supply of food is inadequate or in which draft animals are too expensive for widespread use may hold different views. In Europe, it is possible that public opinion will change if methods become available to enhance the health of livestock, to make livestock resistant to viral infections such as foot and mouth disease, avian influenza or to ensure that livestock could not transmit prion diseases.

AFTER DOLLY – ETHICAL LIMITS TO THE USE OF BIOTECHNOLOGY ON FARM ANIMALS

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The cloning of the sheep Dolly brought to the attention of many people the fact that scientists had made a major breakthrough in their attempt to manage and control life. It also gave rise to a widespread call for ethical limits to the interference with life to be established and enforced. Until recently the main limits to interference with life were of a technical kind: of what it is *possible* to do. Now, and increasingly, scientists are faced with ethical limits: of what it is *acceptable* to do.

Both the increase in power over nature, and the potential increase of speed and efficiency that modern breeding and biotechnology presents, force us to recognise our moral responsibility and to discuss the limits of acceptability. In such discussion ethics provides a way of ensuring systematic and rational reflection on the moral issues raised in the public sphere within an idealised framework of values and principles guiding behaviour.

To the general public animal biotechnology does not exist in a vacuum. It coexists with other uses of biotechnology, primarily within agriculture and the medical area. It therefore makes good sense to discuss, first of all, how in the view of the public animal applications relate to these various uses of biotechnology.

To get a grip on the public understanding two sources are made use of. One consists of the so-called 'Eurobarometer' surveys used by the European Commission to monitor lay perceptions of the new biotechnologies. These surveys have been carried out at regular intervals since 1989. The other source consists of qualitative interviews carried out in Denmark in 2000.

Inspired by the study of public perception of animal biotechnology a number of concerns are formulated and made the subject of closer discussion: utility, risk, animal welfare, animal integrity, environmental concerns, human health, and the fear that there is a "slippery slope" from the use of biotechnology on farm animals to uses on humans.

In the final part of the talk an attempt will be made to formulate different overall views about how to set ethical limits to the use of biotechnology on farm animals. It will be argued that a socially robust application of animal biotechnology must limit uses where problems with animal welfare and animal integrity are likely to occur to applications which are of vital importance, e.g. in biomedical research. For the purpose of farm animal breeding there must be rather narrow limits to the use of biotechnology.

After Dolly – ethical limits to the use of biotechnology on farm animals

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Abstract

The cloning of the sheep Dolly gave rise to a widespread call for limits to the interference with life. Until recently the main limits to the interference with life were of a technical kind: What it is *possible* to do. Now scientists are faced with ethical limits as well: What it is *acceptable* to do.

In this context we see ethics as systematic and rational reflection on the moral issues raised in the public sphere. It is not that the concerns of the general public are necessarily valid, but they are the best point of departure, if the discussion is to lead to a socially robust framework for setting limits to the use of animal biotechnology.

To get a grip on the public understanding two sources are made use of: The Eurobarometer surveys from 1991-2002 and a qualitative interview study carried out in Denmark in 2000. Inspired by these studies of public perception of animal biotechnology a number of concerns are formulated and made the subject of closer discussion, these are risk, animal welfare, animal integrity, and usefulness.

In the final part of the article a principle of proportionality is suggested as the foundation for a socially robust application of animal biotechnology. Only in cases where the usefulness of the technology can be said to outweigh the moral concerns, as in biomedical research, will applications of animal biotechnology stand up to scrutiny in the public sphere.

1. *The cloned sheep, Dolly, made the public aware of animal biotechnology*

Dolly was an unusual sheep. She was in a radical sense fatherless. She originated from a cell taken from the udder of her biological mother. This cell was inserted into a sheep ovum from which the nucleus had been removed, and it was manipulated so that it fused with the 'egg-mass' or cytoplasm of the ovum to form a pre-embryo. The pre-embryo was then inserted into a foster mother who went through a normal albeit closely monitored pregnancy which resulted in the birth of Dolly – the first mammal to be cloned from an adult animal.

Her life was quite different from the lives of other sheep in another way too. Few sheep have names, and if they do, their names are generally known to very few people. Dolly's name, on the other hand, is known by millions of people. Her life and death was part of the international news sphere and today she has become almost a symbol in the public debate on biotechnology. In the main, her fame was and is not based on admiration and fascination. Rather, it is largely the product of public anxiety, and in particular moral concern. This single Dorset ewe brought to many people's attention the fact that scientists had made a major breakthrough in their attempts to manage and control life. She also gave rise to a widespread call for ethical limits to interference with life to be

established and enforced. Until recently, the main limits of this kind of science were technical in nature. The question was: What is it possible to do? Now, and increasingly, scientists face ethical limits. The question has become: What is it *acceptable* to do?

This question of acceptability is an ethical question that can be answered from different points of view. In this paper we will take as our starting point how the European public perceives animal cloning and other applications of biotechnology on farm animal species. In taking this approach we do not assume that the public is always right in matters of ethics. Rather our point is that the problem that faces those who in a European context want to be allowed to make use of animal biotechnology is to get on speaking terms with the rest of society. So by presenting the views of the public within an ethical context it will be possible to make sure that the ethical issues being discussed are the relevant ones from the point of view of a wider public. And the upshot of the ethical discussion presented in this paper will be to formulate the issues that have to be dealt with when it comes to regulation and guidelines for the development and use of animal biotechnology in a European – and probably also a much wider – context.

2. *Biotechnology, animals, ethics and the public*

For the general public animal biotechnology does not exist in a vacuum. It coexists with other uses of biotechnology, primarily within agriculture and the medical area. It therefore makes good sense to discuss, first of all, how animal applications relate to these other uses of biotechnology.

In order to monitor lay perceptions of the new biotechnologies within the EU, the European Commission has carried out regular surveys since 1989 — the most recent being undertaken in 2002. Each of these so-called 'Eurobarometer' surveys consists of simultaneous and identical national surveys of 1000 inhabitants in each member country. The surveys show that wholesale rejection of biotechnology is not very common among Europeans, since many respondents make balanced judgements in which the pros and cons of the different applications of biotechnology are variably assessed (Gaskell, Allum & Stares (eds), 2003; Gaskell & Bauer (eds), 2001; Durant et al. (eds), 1998). They also bring out interesting details of lay thought about biotechnology. In each survey, and in connection with several applications, people are asked to consider the usefulness, risks and moral acceptability of biotechnology, and to give an overall assessment. It turns out that, across Europe, and for all applications, moral acceptability outweighs the other parameters when it comes to explaining the overall assessment of the application. Less surprisingly, perhaps, medical applications tend to be viewed as more acceptable than those within the food sector. Perceived usefulness, risks and moral acceptability all play a role in this distinction. Crucially, however, the usefulness of many medical applications is generally recognised, whereas the usefulness of food applications has yet to be demonstrated to the public.

Application	"Should be encouraged" EU mean score (-2= low; +2=high)
Using genetic testing to detect diseases we might inherit from our parents such as cystic fibrosis/muscoviscidosis/thalassaemia	0,91
Introducing human genes into bacteria to produce medicines or vaccines, for example to produce insulin for diabetes"	0,81
Taking genes from plant species and transferring them into crop plants, to make them more resistant to insect pests	0,34
Developing genetically modified animals for laboratory research studies, such as a mouse that has genes which cause it to develop cancer	-0,07
Use modern biotechnology in the production of foods, for example to make them higher in protein, keep longer or change the taste	-0,11
Introducing human genes into animals to produce organs for human transplants, such as pigs for human heart transplants	-0,22

Table 1: Mean scores in the judgement of to what extent different applications of gene technology should be encouraged in EU in 1996. based on Durant, J. et al (eds), 1998, p.234, 260

As Table 1 shows, the food-medical divide is not the only distinction separating negatively assessed applications from positively assessed ones. Applications of biotechnology involving animals have, it seems, a similar low level of support as applications within the food area: the least supported application at all is xenotransplantation, and the use of research animals enjoys almost as low a level of support as the production of GM foods. The 1999 Eurobarometer survey exhibited similar patterns. The one animal application (i.e. "cloning of animals such as sheep to get milk which can be used to make medicines and vaccines") emerged as having almost as low level of support as the least supported application, GM foods (INRA (Europe), 2000). The 2002 Eurobarometer survey (where the one animal application is xenotransplantation) also shows that although the technology is used for clearly medical purposes, it is given almost as low level of support as the use of biotechnology in the food-sector. It should be mentioned however that the surveys from 1999-2002 show a small but steady increasing support of the use of biotechnology in general and also for the research into, and application of, xenotransplantation.

Application	"Should be encouraged" EU mean score (-2= low; +2=high)
Using genetic testing to detect diseases we might have inherited from our parents, such as cystic fibrosis/mucoviscidosis/thalassaemia	0,67
Introducing human genes into bacteria to produce medicines or vaccines, for example to produce insulin for diabetes	0,51
Developing genetically modified bacteria to clean spillages of oil or dangerous chemicals	0,51
Cloning human cells or tissue to replace sick cells in a patient which are not functioning properly	0,33
Taking genes from plant species and transferring them into crop plants to make them more resistant to pests	0,03
Cloning animals such as sheep whose milk can be used to produce medicines or vaccine	-0,17
Use modern biotechnology in the production of foods, for example to give them a higher protein content, to be able to keep longer or to change the taste	-0,31

Table 2: Mean scores in the judgement of to what extent different applications of gene technology should be encouraged in EU in 1999. (INRA (Europe), 2000)

All three of the applications of animal biotechnology in Table 1 and Table 2 fall within the medical sphere, and yet they enjoy a relatively low level of support. A possible explanation for this is the simple fact that they all involve animals. It is also possible, however, that the specific applications in question — cloning, research animals and xenotransplantation — in themselves evoke negative feelings. Thus cloning is associated in some people's minds with frightening popular images of man-made copies of evil individuals (e.g. in the film 'The Boys from Brazil'). The xenotransplantation of pig hearts into humans is likely to cause unrest because an important barrier between man and animals is thereby breached and blurred. And for many people the use of animals in research calls up an image of cruelty to animals.

Some of the follow-up questions in the 1999 Eurobarometer throw more light on animal cloning. Responses to these questions partly explain the rejection of this particular application. Participants were asked to assess 12 statements about cloning on a five point scale, where 5 = 'strongly agree' and 1 = 'strongly disagree'. The two most strongly supported statements concerned the perceived unnaturalness of cloning. Thus the average score of the statement "even if animal cloning has advantages, it is basically against nature" and "animal cloning threatens the natural order of things"

scored 4.24 and 4.12 respectively. Similarly most agreed (average score 3.85) that animal cloning was simply not necessary, and most disagreed with the statements that animal cloning presents no danger to future generations (average 2.19) and that the risks involved in animal cloning are acceptable (average 2,28) (INRA (Europe), 2000).

The lack of support to animal cloning therefore seems to reflect the fact that ordinary people believe it to be both unnatural, lacking in real usefulness and a genuine risk to mankind. Other medical applications are perceived to be much more useful and much less risky, even though they are also thought of as unnatural.

3. Scepticism does not merely reflect lack of knowledge

It might be supposed that the pronounced public scepticism about forms of biotechnology such as cloning and other animal biotechnologies reflects a low level of understanding of biotechnology among lay observers. According to this so-called 'knowledge deficit' or 'knowledge gap' model, inadequate knowledge leads the public to draw moral conclusions about practices within science that are ill-informed. And since the problem is knowledge, the cure is to feed information into the public sphere more effectively — to *educate* people. Once this is done, public acceptance will follow.

Surveys such as the Eurobarometer have seriously challenged this viewpoint by showing that the question of acceptance or rejection is much more complex than advocates of the deficit model suppose. Eurobarometer surveys include a 'knowledge quiz' in which respondents are asked a series of factual questions about biotechnology. This quiz enables the relationship between optimism about biotechnology and level of knowledge to be examined. The examination, however, reveals that optimism and positive expectations have failed to increase with growing knowledge. In point of fact, it was found in 1997 that, despite a moderate increase in overall knowledge of the subject-matter, optimism about biotechnology had declined in Europe (Biotechnology and the European Public Concerted Action group, 1997). As they acquire more information, people are better able to form an opinion for or against biotechnology — that is, there is a decrease in the number of 'don't knows'. But *pace* the deficit model, they do not acquire a more positive attitude to biotechnology. In particular, there is at best a poor correlation between knowledge and support of individual applications.

The knowledge deficit model has also been challenged at the national level. In Denmark, for example, considerable resources were invested in public information, education and debate about biotechnology in the 1980s. Following this, Danes were among those scoring highest in the Eurobarometer knowledge quiz (Gaskell, Allum & Stares, 2003; INRA (Europe), 2000; Durant et al. (eds), 1998, 2003). But the surveys in which Danish participants displayed their knowledge also show that Danes are among the most critical of biotechnology — which, obviously, is clear evidence against the hypothesis that there is a causal link between knowledge and acceptance.

4. Animals, biotechnology and the public: moving beyond the general picture

Apart from the indications given by the follow-up questions about cloning, the Eurobarometer does not offer any detailed explanation of the motives and reasoning lying behind critical attitudes to the mix of animals and biotechnology. To add more detail to the picture painted by the Eurobarometer, a series of focus group interviews were carried out in Denmark in 2000. In the course of these

interviews both food and non-food applications of biotechnology were examined. In both cases, a number of animal applications were reviewed.

Generally the interviews reproduced the picture generated by the Eurobarometer surveys — i.e. confirmed that food applications are assessed much more negatively than non-food applications, and that medical applications are assessed most positively. To many Danes, indeed, the border between food and medical applications virtually constituted a boundary between the unacceptable and the acceptable, although in no case did an interviewee approve of carte blanche being given to scientists working in biomedicine.

4.1 Risks

The discussion revealed that people are concerned with risks to the environment as well as to human health. It is mainly applications within agriculture and food production that give rise to images of risk; whereas applications of biotechnology on animals, including farm animal species, within biomedical research plays a remarkably insignificant role in people's perception of risk.

Although a general fear of eating GM foods was not displayed, health risks were an important theme in the food discussions. Concerns about eating genetically manipulated foods related, not to dangers of the here and now, but to long-term risks. Health risks attaching to the consumption of manipulated meat were only mentioned occasionally, but it must be assumed that general unease about GM food will extend to products originating from genetically modified farm animals. Also incorporated in this unease is a concern that at present 'we' lack sufficient knowledge to make predictions. The 'we' here, as elsewhere, seems to relate to the scientific community and health experts. It records a perception of scientific uncertainty. Such arguments are fortified with experiences drawn from other contested issues, such as the issue of BSE. During the period of the interviews, and following the discovery of an infected cow in Denmark, BSE was widely discussed in Denmark. This gave rise to the fear that scientists are unable to control the consequences of what they are doing.

As regards the risk to human health, animal biotechnology is viewed as being on par with applications of biotechnology to plants and micro-organisms. This contrasts with the attitude to environmental risks, where some argue that modified animals pose less of a problem. GM animals can be caught and slaughtered if necessary and thus there is less reason to worry about potential problems caused by any uncontrolled spread of 'unwanted genes'. These arguments claim that since animals are larger and slower, they are also easier to control.

Occasionally, arguments about risk are supported with reference to the perceived unnaturalness of the new biotechnologies. In such arguments — which are usually expressed in general terms and relate to genetic modification as such — the idea is that, by manipulating animals, we challenge the natural or God-given order of things and thus bypass certain (not always specified) safety mechanisms that are inherent in nature. These safety mechanisms are often associated with barriers between species and the inherent slowness of traditional breeding and propagation techniques.

Other qualitative interviews have demonstrated that, in contrast to widespread concern about its health risks within the scientific community, xenotransplantation is only discussed in terms of risk by lay people to a limited extent (Dahl et al., 2002). Similarly, these interviews demonstrate that

perception of risk plays no direct role in negative lay perceptions of the use genetically modified research animals.

Finally it should be mentioned the biotechnologies that are now being used on animals is often seen as an augury of what later will be used on humans. The debate around the cloning of Dolly is a school example of this. It very quickly turned from the ethical problems arising form the cloning of animals to the problems arising from the same technology applied to humans. It is noteworthy that most western countries very quickly legislated against the cloning of human cells where as only few countries to date has made legislation concerning the use of cloning on animals. The implication seems to be that to many the use of biotechnology on animals is the starting point of a "slippery slope" that will end up with the same technologies being used on humans.

4.2 Animal welfare

Animal welfare, and especially the welfare of domestic animals, has been a major issue in Denmark over recent decades. Strikingly, however, while the welfare of domestic and research animals have been widely discussed, the welfare problems of genetically modified or cloned animals has largely been ignored in the public debate. This disparity is expressed in the interviews when discussion touches on the domestic animals: the issue of animal welfare is only taken up spontaneously in two of the interviews, and in both cases the interviewees referred to animal welfare problems in cows engineered to produce more milk — doubtless, a reflection of the fact that this issue is the only one which has been the focus of public debate over animal welfare issues and biotechnology.

Although the suffering of genetically modified research animals has so far not become a public issue, it does come up in the focus groups. This might be because animal suffering is one of the issues — perhaps even the major issue — raised quite generally by the use of animals for research. Here, as in debates over transgenic research animals, it should be noted that it is often hard to separate situations in which research animals are generally disapproved of and situations in which negative feelings are evoked by a special factor, such as the fact that the animals are transgenic.

4.3 Other moral arguments

Another group of concerns (closely related to concerns about animal welfare) has to do with the place of animals in the hierarchy of nature. Thus some interviewees implied that arguments capable of justifying the application of biotechnology to higher organisms would need to be better than those required where micro-organisms and plants are at issue.

Typically, in these classifications, the more like us the animals are, the harder it becomes to justify applications of biotechnology. Fish are not as important as calves, for example, and primates are pre-eminent. The idea of xenotransplantation evokes a particularly negative feeling (Dahl et al., 2002) and some interviewees cannot imagine walking around with the organs of another animal. This feeling tends to be stronger when the organ is a heart and the donor is a pig — perhaps because pigs have traditionally been thought to represent the very opposite of human purity and sophistication.

These concerns often seem less palpable than concerns about risks and animal welfare, bur none the less they play an important role. Often these concerns are expressed in the terms of borders and limits. They indicate that to some people there are limits to the degree of control that humans can

ethically legitimate to exercise over other living beings regardless of the consequences to human and animal welfare. Concepts as integrity and dignity are often evoked to describe these limits. There is a tendency in the academic literature to write of these concerns as irrational and/or religious, but lack of conceptual clarity should not make one dismiss them as relevant factors in the discussion too quickly. The idea of nature having some sort of integrity (which we will say something about in Section 5) seems to be rooted in very common everyday experiences of nature and express a view of nature that goes beyond a purely scientific one. The concept is certainly complex and ambiguous and in need of further reflection, but nonetheless seems to be very important, if one is to understand the perception of biotechnology by the public (Cooper 1998).

4.4 Usefulness

In public debate about biotechnology the predominant tone is often negative — the question often being whether this or that application should be rejected. A less negative attitude comes into the foreground when people are asked to consider the usefulness of biotechnologies. However, the concept of usefulness is complex. It is a contested concept.

Three interpretations of usefulness can be identified in the biotechnology debate. Each was reflected at some point in the interviews. Some observers — primarily representatives of the biotech industry and others with vested interests in biotechnology — defend an *economic* usefulness argument, according to which biotechnology is useful in technical and financial terms. This notion was not shared by many of the participants in the interviews. Indeed some stated that making money is, on its own, not a sufficient argument for such controversial technologies.

The *societal* usefulness argument is in keeping with this latter attitude. It is commonly put directly, or gestured at, in the interviews. The main idea here is that biotechnology is useful, and to that extent more acceptable, when it serves societal needs that amount to more than mere economic gain. The final kind of usefulness concerns usefulness to oneself or one's close relatives. This (as we might call it) *self-interested* usefulness often generates serious dilemmas in which abstract, objective assessments clash with highly contextualised, subjective assessments involving the health, life and death of oneself or a close relative.

In general societal usefulness relates primarily to the avoidance of risk, the management or elimination of disease, and the alleviation of third-world poverty. In relation to domesticated animals, the transgenic pig with reduced fat is sometimes put forward as an example of a useful application, because it addresses a recognised health problem, obesity, and thus introduces a health benefit to consumers. Those advancing this argument seldom do so with reference to themselves. They focus instead on obesity as a societal problem.

Others, however, argue that functional foods in general are the wrong strategy for solving problems connected with the composition of a person's diet. Both within the medical sphere and in relation to domesticated animals, it is often argued that biotechnology is the wrong way to pursue laudable goals. Alternative strategies should be pursued or examined.

In the medical sphere, debates about animals primarily concerned their use in research. Often, these debates involved a dilemma between societal or self-interested usefulness, on the one hand, and the general uneasiness about the use of biotechnology and animals, on the other. Like the medical

applications of biotechnology to which they lead, research animals were looked upon positively by many interviewees because their use may help us to relieve the pain and suffering of the sick. This kind of argument is, however, often presented with a disclaimer — a disclaimer in which it is stressed that however useful they are, research animals are still problematic. The following highly ambivalent statement from a male participant, replying to a question whether research animals are acceptable, is a nice illustration of this: *"Yes and no. I feel sorry for the animals that they have to pay. But if we don't have other possibilities then we have to try it. Apparently there are no other possibilities — then you have to do something to produce these products to help cancer patients and whatever. Then it's OK, but still I believe it's wrong."*

As mentioned above, it is quite commonly thought that, although transgenic research animals are unacceptable, sufficient benefits to mankind can justify them. A number of arguments against transgenic research animals were presented in which the individual (as a citizen) tried to weigh the pros and cons at a societal level. But the individuals who put these arguments often confessed that, when they had to assess biotechnological applications from a strictly individual point of view, thinking of themselves as potential beneficiaries of the technology they had rejected, they were inclined to adopt a more lenient attitude.

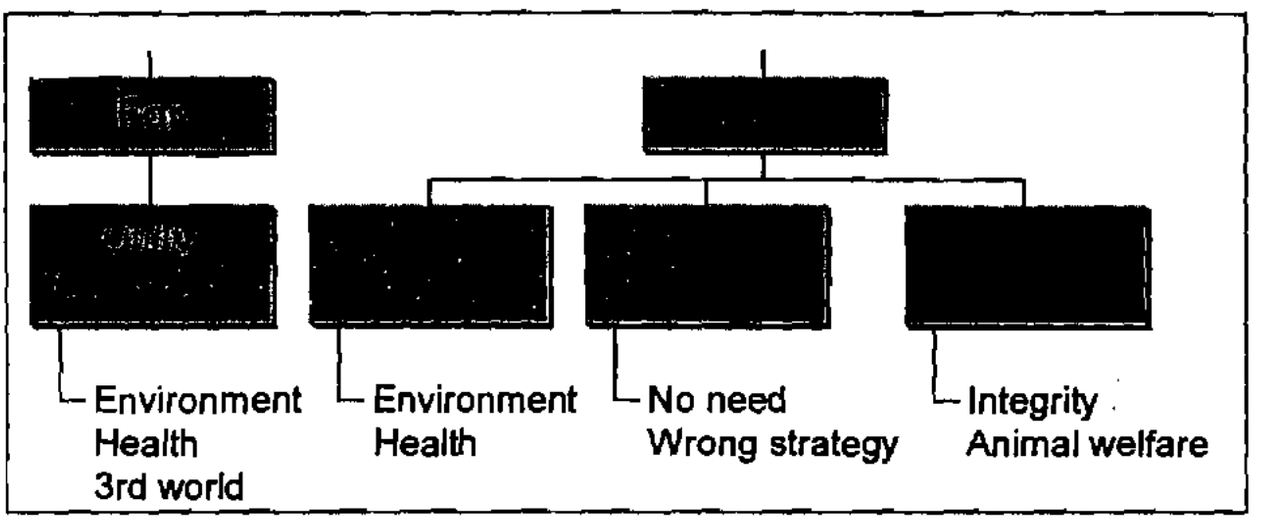

Figure 1: Overview of arguments for and against animal biotechnology offered by members of the Danish public.

5. Ethical limits to the use of biotechnology on animals?

In this article we have tried to show what kind of questions and worries the use of biotechnology on farm animals evokes in the European public both when it concerns farm animals as species and as production units in the agricultural sector. They can roughly speaking be said to be a list of the ethical questions that a society as Denmark has to discuss when considering the ethical aspects of biotechnology and establishing a framework for the use of it. At least if the discussion is to be informed by the ethical values held by large parts of the society. There are two main reasons why this approach would be preferable.

First of all it is in tune with the democratic tradition of Western Europe that legislation and regulation of ethically sensitive issues is based on thorough public debate and consensus seeking policies that try to integrate the ethical questions raised. Secondly it seem obvious that the lesson to be learned from the debate over GM-crops and the subsequent consumer reactions in Western Europe is that it is a necessary requisite for the successful implementation of biotechnology in a society like the Danish that the ethical doubts of the public are taken seriously when the technology is introduced.

It would be premature to say that a thorough public debate on the use of biotechnology on farm animals has taken place in Denmark or any other western country. But if we take the ethical questions and worries described in this article as a rough picture of the ethical considerations that will emerge from such a debate, the following framework for the use of the technology can be drawn. It should be noted at this point that establishing the framework for applications of biotechnology on farm animals only constitutes the first step in the very complex regulation and legislation process. In this article however, we concentrate on the general framework, since the discussion of how actually to regulate and legislate would lead to far in this context

First of all the framework has to accommodate the difference between the public and the scientific conception of risks, both to human health and to the environment that can be found in many areas of the debate on biotechnology (Hansen et al. 2003). Especially the public conception of scientific uncertainty, lack of trust in experts and public distrust to all too firm reassurances about safety need to be addressed.

Secondly it is necessary to take the question of animal welfare very seriously. Although it has not yet figured as a predominant subject specifically in the discussion about the use of biotechnology on farm animals there can be no doubt that the notion plays an important and even increasing role in the general discussion about farm animal production.

Thirdly the notion of other moral concerns than threats to human, environmental and animal welfare has to be respected. These concerns can be about social justice (who will benefit from the technology?), the relationship between expert and public influence on legislation and regulation, and about the consequences of the technology for the understanding of the relationship between man and nature. However, in this article we will limit ourselves to a short description of the concerns about biotechnology's possible violation of the integrity of animals. This concern differs from the risk and welfare concerns mentioned above in that violation of integrity cannot be sustained by empirical evidence in the same way as for example environmental risks or welfare problems can. However, it is still possible to exemplify what is at stake.

One example that many people react to is the case of the blind hens. A Canadian scientist involved in poultry breeding bred a blind egg-laying hen. This hen according to the researcher would serve to reduce the welfare problems of free-range chickens that inflict a lot of pain on each other by pecking and sometimes even cannibalising the weaker members of the flock. The blindness was thus not inflicted on the chickens, but something they were born with and from welfare perspective they are better of than their seeing peers. Still the notion of deliberately breeding blind hens to solve production problems in the agricultural sector stirs a lot emotions and resentment in the general public. Something just seems to be wrong when you deliberately create an animal with less potential than a normal animal of the same kind. This is not the place to discuss the many different

ways that the idea of blinding hens to improve their welfare can be defended or attacked. But it is obvious from the data presented here that for many people there may be something inherently ethically troublesome about using biotechnology to change the nature of animals, whether it hurts them or not

Finally a key point in the discussion concerning the ethical acceptability of animal biotechnology is the notion of utility. This will call for a balancing of the possible ethical good outcomes of the use of biotechnology on farm animals against the ethical wrongs that this use also entails. This can be clearly seen in the relatively sharp distinction that was found between medical and food oriented uses of the technology. In the first case the good aspects are often seen as outweighing the bad, whereas the opposite is true in the second case. If the goal is deemed important (ethically good) enough the evil that it entails is accepted as a sort of necessary evil. This weighing of the ethical scales is of course a complex and multi-facetted discipline that depends upon both the specific use of biotechnology in question and the person who is performing it. But taken up on a societal scale it clearly seems that in general the use of biotechnology on farm animals is something that needs to be argued rather than the other way around where those opposing it, are left with the burden of evidence.

To sum up it seems that a socially robust application of animal biotechnology must limit uses where problems with animal welfare and animal integrity are likely to occur to applications which are of vital importance, e.g. in biomedical research. For the purpose of farm animal breeding there must be rather narrow limits to the use of biotechnology. A principle of proportionality where each application of biotechnology is subjected to scientific and ethical discussion before either endorsed or rejected seems to be the ethically most viable way to go, if the technology is to enjoy broad appreciation in the general public. It should be mentioned that his principle has in fact been suggested as the governing principle in the guidelines for applications of biotechnology to animals in Denmark by a governmentally appointed committee (Ministeriet for Videnskab, Teknologi og udvikling 2003).

This way of interpreting the concerns identified in this article and establishing a framework for the application of the technology is perhaps more limiting than most scientists working within the field would like. They may have hoped for more. But if we look at the attitudes of the general public it seems that aspiring for very broad applications implies a risk that the general public will turn down all uses of the technology – as we saw in the GM-food debate in Europe during the 1990'ies. By taking heed of the principle of proportionality one might have to rule out several possible applications of biotechnology to farm animals, but it ensures that the ones left will also be acceptable to the general public – and that is both from a scientific and a societal perspective to be preferred.

6. Conclusion

The European public is worried about animal cloning and other forms of animal biotechnology. However, contrary to a widespread belief in the scientific community, this worry cannot simply be explained in terms of a lack or distortion of factual information. To create a socially robust framework for the discussion of the ethical limits concerning the use of biotechnology on animals, we believe that one should take these worries as the point of departure. It is not that they can stand

alone or uncritically be implemented. They have to be thoroughly discussed. But to do that it is important to have a real understanding of them. The key items on this agenda concern risk, animal welfare, usefulness and other moral concepts. But in connection with each of these items the public and the scientists may well conceive of the problems differently. Thus the public typically view risks in a broader and more long-term perspective than scientists. They also believe that if usefulness is to serve as a positive argument, it must involve more than mere commercial success. It follows that, if European scientists want to be able to use animal biotechnology in their research with public support, they must broaden their conception of what matters beyond the idea of a scientifically measurable risk and they must narrow their notion of usefulness so that the mere possibility of making a profit is not enough to justify animal biotechnology. We suggest that a principle of proportionality is made the basis of the ethical evaluation of the use of biotechnology on farm animals. This basically means that all use of biotechnology must be justified by arguing why it is necessary in a specific case to transgress the ethical borders that the concepts of risk and animal welfare and integrity raises. By limiting the use of biotechnology in this way we foresee that many of the controversies surrounding other uses of biotechnology (e.g. GM-crops) can be avoided.

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Literature

Biotechnology and the European Public Concerted Action group (1997): Europe ambivalent on biotechnology. Nature. Vol. 387, pp. 845-7.

Cooper, David E. (1998): Intervention, humility and animal integrity, in Holland, Allan & Johnson, Andrew (eds.): *Animal Biotechnology and Ethics*, Chapman&Hall, London, pp. 145-155.

Dahl, K., Sandøe, P., Johnsen, P.F., Lassen, J. & Hansen A.K. (2002): Outline of a risk assessment: the welfare of future xeno-donor pigs. Animal Welfare, in press.

Durant, J., Bauer, M.W. & Gaskell, G. (eds.) (1998): Biotechnology in the public sphere – a European sourcebook. Science Museum. London.

Gaskell, G. & Bauer, M.W. (eds.) (2001): Biotechnology 1996-2000 – the years of controversy. Science Museum. London.

Gaskell, G., Allum, N. & Stares, S.: Europeans and Biotechnology in 2002. Eurobarometer 58.0. 2. ed. March 21st 2003). http://europa.eu.int/comm/public_opinion/archives/eb/ebs_177_en.pdf

Hansen, J., Holm, L., Frewer, L., Robinson. P., Sandøe, P. (2003): Beyond the knowledge deficit. Recent research into lay and expert attitudes to food risks. *Appetite* 41: 11-121

Ministeriet for Videnskab, Teknologi og udvikling (2003): Genmodificerede og klonede dyr. København

INRA (Europe) (2000): Eurobarometre 52.1. Les Europeens et la biotechnologie. 15. mars 2000.

Lassen, J., Madsen, K.H & Sandøe, P. (2002): Ethics and genetic engineering – lessons to be learned from GM foods. Bioprocess and Biosystems Engineering 24(5), p.263-71.

Madsen, K.H., Holm, P.B., Lassen, J. & Sandøe, P. (2002): Ranking genetically modified plants according to familiarity. Journal of Agricultural and Environmental Ethics, in press.

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