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VITRIFICACIÓN DE OVOCITOS BOVINOS MEDIANTE LA TÉCNICA OPEN PULLED STRAW: ESTUDIO ESTRUCTURAL DE CROMOSOMAS, MICROTÚBULOS Y MICROFILAMENTOS Y POSTERIOR DESARROLLO EMBRIONARIO IN VITRO

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ÍNDICE

CAPÍTULO I. INTRODUCCIÓN Y OBJETIVOS.. 1

CAPÍTULO II. REVISIÓN BIBLIOGRÁFICA.. 11

2.1. Maduración nuclear y citoplasmática del ovocito.. 13

2.1.1. Bloqueo meiótico y reinicio de la meiosis......... 14

2.1.2. Inhibición del reinicio de la meiosis. 16

2.2. Principios generales de crioconservación.. 19

2.1. Congelación lenta.. 20

2.2 . Congelación rápida y ultrarrápida.. 22

2.3. Crioconservación de ovocitos... 23

2.3.1. Efectos del enfriamiento y la congelación sobre las estructuras ovocitarias.. 24

2.3.1.1. Efectos de la temperatura sobre la placa metafásica................................ 24

2.3.1.2. Efectos de la temperatura sobre el citoesqueleto ovocitario...................... 27

2.3.1.3. Efectos de la temperatura sobre otras estructuras ovocitarias................... 28

2.4. Vitrificación.. 29

2.4.1. Factores que influyen en la vitrificación... 30

2.4.1.1. Velocidades de congelación y descongelación.. 30

2.4.1.2. Crioprotectores y aditivos crioprotectores.. 30

2.4.1.2.1. Crioprotectores permeables.. 31

2.4.1.2.2. Crioprotectores no permeables. 32

2.4.1.3. Estadios de maduración. 33

2.4.1.4. Soportes para vitrificación. 35

2.5. Análisis comparativo entre ovocitos de animales prepúberes y adultos.. 36

2.6. Bibliografía... 39

CAPÍTULO III. Effects of vitrification in open pulled straws on the cytology of *in vitro* matured prepubertal and adult bovine oocytes………. 51

3.1. Abstract... 53

3.2. Introduction... 53

3.3. Materials and Methods...… 55

3.4. Results..……………………… 60

3.5. Discussion... 62

3.6. Bibliography... 66

CAPÍTULO IV. Effects of roscovitine on the nuclear and cytoskeletal components of calf oocytes and their subsequent development.. 69

4.1. Abstract.. 71

4.2. Introduction.. 71

4.3. Materials and Methods.. 73

4.4. Results.. 77

4.5. Discussion.. 85

4.6. Bibliography... 90

CAPÍTULO V. Vitrification of calf oocytes: effect of stage of maturation and prematuration on the nuclear and cytoskeletal components of calf oocytes and their subsequent development.................. 93

5.1. Abstract.. 95

5.2. Introduction.. 95

5.3. Materials and Methods.. 97

5.4. Results.. 104

5.5. Discussion.. 109

5.6. Bibliography... 113

CAPÍTULO VI. DISCUSIÓN GENERAL.. 119

CAPITULO VII. CONCLUSIONES.. 131

CAPÍTULO I:
INTRODUCCIÓN Y OBJETIVOS.

Los ovocitos bovinos recuperados de ovarios de matadero se han convertido en una fuente ampliamente utilizada para procedimientos tales como la fecundación *in vitro*, la clonación u otras tecnologías reproductivas relacionadas. Debido a que el ovocito sólo permanece viable un periodo limitado de tiempo y a que el número de ovocitos que puede ser recogido en un día determinado es limitado, la capacidad para mantener a los ovocitos crioconservados incrementaría enormemente su utilidad en la investigación básica así como en las aplicaciones comerciales. Sin embargo, y hasta la actualidad, la capacidad de desarrollo de los ovocitos que han sido congelados, medida como el número de embriones viables por ovocito congelado, se mantiene baja comparada con la obtenida a partir de ovocitos frescos.

En la actualidad ya es posible crioconservar ovocitos y embriones de algunas especies de mamíferos mediante los protocolos de congelación lenta, congelación rápida y vitrificación. Sin embargo, y a pesar de los esfuerzos realizados por numerosos grupos de investigación, son pocos los trabajos que documenten gestaciones y nacidos vivos a partir de ovocitos congelados (Fuku *et al.*, 1992; Hamano *et al.*, 1992; Otoi *et al.*, 1992; Suzuki *et al.*, 1996; Vieira *et al.*, 2002).

La vitrificación, descrita inicialmente en embriones por Rall y Fahy (1985), corresponde a una técnica de congelación ultrarrápida basada en el contacto directo entre la solución de vitrificación que contiene los agentes crioprotectores y ovocitos o embriones con el nitrógeno líquido. La definición física de la vitrificación es la solidificación de una solución a baja temperatura sin que ésta llegue a cristalizar debido a un enorme incremento de la viscosidad (Fahy, 1986), manteniendo así la distribución molecular e iónica que existía antes de la congelación (Fahy *et al.*, 1984).

La estrategia de la vitrificación es básicamente diferente a la estrategia de la congelación lenta. Una velocidad lenta de congelación intenta mantener un delicado balance entre varios factores los cuales pueden resultar en lesiones celulares provocadas por la formación de cristales de hielo, los choques osmóticos, el efecto tóxico de los crioprotectores, la concentración de electrolitos intracelulares, los daños por enfriamiento, las fracturas en la zona pelúcida y las alteraciones de los organelos intracelulares, el citoesqueleto o el contacto entre células (Massip *et al.*, 1995;

Dobrinsky, 1996; Kasai, 1996; Martino *et al.*, 1996; Saha *et al.*, 1996). La vitrificación elimina totalmente la formación de cristales de hielo ya que al aumentar la velocidad de congelación disminuyen los daños causados por el enfriamiento pasando rápidamente por la zona de mayor peligro que está situada entre los +15º y los –5ºC (Dobrinsky, 1996; Martino *et al.*, 1996; Isachenko *et al.*, 1998).

El proceso de vitrificación requiere la presencia de una alta concentración de crioprotectores, por lo que es necesario minimizar el daño celular provocado por el estrés osmótico o la toxicidad química provocada por dichas concentraciones utilizadas. Por ello, el principal objetivo de un protocolo de vitrificación debe ser la disminución de la toxicidad sin una pérdida de la efectividad de los agentes crioprotectores (Liebermann *et al.*, 2002). Para reducir la toxicidad de las soluciones crioprotectoras se han desarrollado diferentes protocolos donde se combinan uno o varios crioprotectores que se suplementan con azúcares, macromoléculas, etc. En el bovino, el contacto de los ovocitos con etilenglicol (Martino *et al.*, 1996; Saunders y Parks, 1999) provocó daños estructurales a nivel de huso cromosómico y filamentos de actina así como una disminución de los ovocitos que alcanzaron el estadio de blastocisto.

Otro de los factores a considerar en el proceso de vitrificación son los estadios de maduración meiótica de los ovocitos, los cuales también pueden influir en su capacidad para sobrevivir a la criopreservación. La congelación de ovocitos en estadio de vesícula germinal podría tener ventajas ya que los microtúbulos aún no están organizados en forma de huso y el material genético está dentro del núcleo protegido por la membrana nuclear. Sin embargo, los índices de supervivencia de ovocitos vitrificados en vesícula germinal son todavía muy bajos en el vacuno (Suzuki *et al.*, 1996; Vieira *et al.*, 2002) y porcino (Didion *et al.*, 1990; Isachenko *et al.*, 1998) y podría ser debido a que el proceso de congelación dañaría las membranas plasmáticas o la comunicación intercelular entre el ovocito y las células del cúmulus perjudicando así su posterior maduración (Hochi *et al.*, 1998).

El índice más alto de blastocistos obtenidos tras un proceso de vitrificación es a partir de ovocitos bovinos criopreservados en metafase de la segunda división meiótica (25% blastocistos: Vajta *et al.*, (1998)). Algunas de las posibles causas del bajo porcentaje de

blastocistos obtenidos en los estudios de vitrificación de ovocitos en estadio de metafase II podría deberse al daño estructural irreversible provocado por la criopreservación en la membrana del ovocito (Arav y Zeron, 1997), en la organización del huso con las consecuentes alteraciones de los cromosomas en los ovocitos en metafase II (Hochi *et al.*, 1998; Saunders y Parks, 1999; Chen *et al.*, 2001), en la distribución de los gránulos corticales (Hyttel *et al.*, 2000) o en la distribución de los filamentos de actina (Hotamisligil *et al.*, 1996; Saunders y Parks, 1999). Existen algunos trabajos en los que se ha planteado la posibilidad de congelar en estadios intermedios de desarrollo, como por ejemplo, vesícula germinal rota (Men *et al.*, 2002) o en el periodo entre metafase I y II (Le Gal y Massip, 1999) con un relativo éxito.

Un intento para mejorar los porcentajes de supervivencia y desarrollo embrionario de los ovocitos tras su vitrificación consistiría en premadurar citoplasmáticamente al ovocito antes de ser crioconservado. De esta forma, el ovocito podría ser congelado en un estadio inmaduro, estadio donde las estructuras cromosómicas y microtubulares estarían más protegidas, mientras que su competencia citoplasmática para desarrollarse tras la congelación se vería incrementada. Esta premaduración podría conseguirse tras un cultivo previo de los ovocitos con inhibidores de las quinasas dependientes de ciclinas como pueden ser la roscovitina o la butirolactona I (Mermillod *et al.*, 2000; Lonergan *et al.*, 2003). Mermillod et al. (2000) bloquearon el reinicio de la meiosis de ovocitos bovinos durante 24 horas con un concentración 50 µM de roscovitina sin que ello provocara una disminución en la capacidad de desarrollo de los embriones obtenidos tras el tratamiento.

Para que un proceso de vitrificación tenga éxito, las muestras deben entrar en contacto con el nitrógeno líquido (-196ºC) lo antes posible por lo que se debe minimizar el tiempo en que la muestra entra en contacto con los vapores de nitrógeno (-180ºC) que se producen al introducirla en el nitrógeno líquido. Por tanto, el tamaño de la muestra debe ser lo más pequeño posible para que la capa de vapor que se forma al contacto con el nitrógeno líquido sea mínima y la velocidad de congelación se vea aumentada (Liebermann y Tucker, 2002). Para minimizar el volumen de la solución de vitrificación se han descrito diferentes tipos de soportes físicos. Entre ellos podemos citar soportes como *open pulled straw*, *closed pulled straw*, *flexipet-denuding pipette*, microgotas,

gradillas de cobre de microscopía electrónica, sistema *hemistraw, nylon mesh y cryoloop* (Vajta *et al.*, 1998; Chen *et al.*, 2001; Liebermann y Tucker, 2002).

La posibilidad de utilizar los ovocitos procedentes de animales prepúberes sería de gran utilidad en los programas de mejora genética debido a que permiten acortar el intérvalo generacional y, en consecuencia, aumentar la presión de selección. Sin embargo, son varios los estudios que concluyen que los ovocitos procedentes de animales prepúberes son menos competentes para desarrollarse tras la fecundación que aquellos provenientes de animales adultos (revisado por Gandolfi *et al.*, 2000). Si bien los ovocitos de vacas prepúberes presentan porcentajes de división embrionaria parecidos a los obtenidos en vacas, el desarrollo embrionario de estos ovocitos se mantiene bajo respecto al obtenido con ovocitos de vacas adultas (Khatir *et al.*, 1998). Esta baja eficiencia se atribuye a cambios en la distribución de los gránulos corticales (Duby *et al.*, 1996), a retrasos en la formación del aster espermático (Damiani *et al.*, 1996), a un menor diámetro celular (Armstrong, 2001) o a un deficiente metabolismo energético, enzimático o proteico (revisado por Gandolfi *et al.*, 2000), resultados que sugieren un retraso o una deficiente maduración citoplasmática *in vitro*.

Así, teniendo en cuenta las consideraciones anteriores, los objetivos de esta tesis doctoral fueron los siguientes:

1. Evaluar los efectos de vitrificar ovocitos de vaca y ternera mediante la técnica *open pulled straw* sobre el huso cromosómico y el citoesqueleto.
2. Valorar el desarrollo embrionario *in vitro* de ovocitos de vaca y ternera vitrificados mediante la técnica *open pulled straw*.
3. Analizar la efectividad de diferentes concentraciones de roscovitina para inhibir el reinicio de la meiosis en ovocitos de ternera durante 24 horas y analizar su efecto sobre el huso cromosómico, el citoesqueleto y el posterior desarrollo embrionario.
4. Determinar la influencia del estadio de maduración (vesícula germinal rota *vs.* metafase de la segunda división meiótica) de ovocitos de ternera en el momento de ser vitrificados por *open pulled straw* sobre el huso cromosómico, el citoesqueleto y el posterior desarrollo embrionario *in vitro*.

5. Evaluar los efectos de una premaduración con roscovitina previa a la vitrificación por *open pulled straw* de ovocitos de ternera sobre el huso cromosómico, el citoesqueleto y el desarrollo embrionario *in vitro*.

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CAPÍTULO II:
REVISIÓN BIBLIOGRÁFICA.

2.1. Maduración nuclear y citoplasmática del ovocito.

Al nacimiento, los ovocitos de las especies mamíferas están bloqueados en la fase G2 de la profase de la primera división meiótica (Wassarman y Albertini, 1994) y tienen que reiniciar y completar la meiosis y la maduración para que la fecundación pueda llevarse a cabo. La competencia meiótica, es decir, la capacidad de los ovocitos para reiniciar y completar la meiosis, se adquiere progresivamente durante el crecimiento folicular y ovocitario y está asociada a una serie de cambios nucleares y citoplasmáticos (Sorensen y Wassarman, 1976; Pavlok *et al.*, 1992; Lonergan *et al.*, 1994; De Smedt *et al.*, 1995; Eppig, 1996).

Los principales cambios nucleares incluyen la ruptura de la vesícula germinal (GVBD), condensación cromosómica y progresión a metafase I (MI), extrusión del primer corpúsculo polar y bloqueo en la metafase de la segunda división meiótica (MII). *In vivo,* el reinicio de la meiosis es provocado por el pico preovulatorio de LH. *In vitro*, el reinicio de la meiosis y la maduración tienen lugar de manera espontánea después de retirar físicamente al ovocito del folículo (Pincus y Enzmann, 1935; Edwards, 1965; Moor, 1988).

En los ovocitos de conejo (Jelinkova *et al.*, 1994), cerdo (Hirao *et al.*, 1995), mono rhesus (Schramm *et al.*, 1993) y vaca (Lonergan *et al.*, 1994; Fair *et al.*, 1995), se ha observado que la competencia meiótica está relacionada con el tamaño folicular. Así, en el bovino se ha observado que sólo aquellos ovocitos que provienen de folículos de un diámetro superior a 2 mm son meióticamente competentes (Fair *et al.*, 1995). Por el contrario, aquellos ovocitos con un diámetro inferior o los ovocitos corticales no adquieren totalmente la competencia meiótica. De hecho, algunos de estos ovocitos son capaces de realizar una meiosis parcial llegando hasta MI, pero pocos son capaces de llegar hasta MII y, menos aún, de desarrollarse hasta estadio de blastocisto (Arlotto *et al.*, 1996).

Durante la maduración citoplasmática existe una reorganización de los organelos citoplasmáticos (Hyttel *et al.*, 1986; Shamsuddin *et al.*, 1993; Ducibelia *et al.*, 1994; Caralco, 1995), comienza la síntesis de proteínas específicas (Moor y Crosby, 1986; Sirard *et al.*, 1989; Tatemoto y Horiuchi, 1995; Wu *et al.*, 1996) y se produce un

incremento de la actividad de las quinasas, iniciándose complejas cascadas de fosforilación y desfosforilación de proteínas específicas que involucran a numerosas quinasas como el *metaphase promoting factor* (MPF), la familia de las *mitogen-activated protein kinases* (MAPK, MAPKK, MAPKKK), el factor citostático (CSF), el AMPc y el receptor del *epidermal growth factor* (EGFR). Se cree que estas cascadas de fosforilación activan moléculas reguladoras nucleares y ooplasmáticas (Motlik y Rimkevicova, 1990; Collas *et al.*, 1993; Gall *et al.*, 1993; Goren y Dekel, 1994; Goren *et al.*, 1994; Gotoh y Nishida, 1995; Lèvesque y Sirard, 1995; Tatemoto y Terada, 1995; Fissore *et al.*, 1996). Todos estos aspectos determinan que la maduración citoplasmática del ovocito no pueda ser observada por técnicas sencillas, pero que pueda ser indirectamente evaluada valorando la capacidad del ovocito para ser fecundado y desarrollarse *in vitro* hasta el estadio de blastocisto.

2.1.1. Bloqueo meiótico y reinicio de la meiosis.

In vivo, el pico preovulatorio de LH libera al ovocito de su primer bloqueo meiótico conduciéndolo a su segundo bloqueo meiótico en MII. La progresión desde el primer al segundo bloqueo meiótico se denomina maduración ovocitaria y a partir de aquí, el ovocito está listo para ser ovulado y fecundado. Producto de la fecundación se produce la activación del ovocito que le provocará la salida del segundo bloqueo meiótico.

Los mecanismos por los cuales se produce el bloqueo meiótico y su reinicio no están claramente establecidos, pero probablemente dependen de factores procedentes de las células foliculares (Faerge *et al.*, 2001). Entre estos factores foliculares podemos mencionar al AMPc (Tornell y Hillensjo, 1993) y las purinas. Se ha sugerido que el AMPc producido por las células de la granulosa y transportado al ovocito es el responsable de la inhibición de la meiosis *in vivo* (Dekel, 1988). La fosforilación de las proteínas mediante la proteína quinasa A dependiente de AMPc (PKA) parece ser el mecanismo de acción por el cual el AMPc inhibe el reinicio de la meiosis (Heikinheimo y Gibbons, 1998).

Durante la maduración nuclear se produce un incremento de la actividad de las quinasas, iniciándose cascadas específicas de fosforilación y desfosforilación del MPF (*maturation-promoting factor o metaphase-promoting factor*) y la actuación de la

MAPK (*mitogen-activated protein kinase*), los cuales son en último término los responsables de la progresión meiótica (Nurse, 1990; Gotoh y Nishida, 1995), siendo esta última quinasa un factor crítico en la regulación del inicio de la meiosis (Motlik y Rimkevicova, 1990).

El MPF es un miembro del grupo de las quinasas dependientes de ciclinas. Esta serina treonina quinasa está formada por la unidad catalítica $p34^{cdc2}$, homóloga a la proteína quinasa cdc2 de la levadura, y por la subunidad reguladora ciclina B (Dunphy *et al.*, 1988; Murray *et al.*, 1989). Durante la fase de crecimiento de los ovocitos, las dos subunidades forman un preMPF fosforilado en los residuos treonina 161, treonina 14 y tirosina 15 de la subunidad $p34^{cdc2}$. Al inicio de la maduración nuclear, se activa el dímero a través de la desfosforilación específica del residuo tirosina 15. Esta desfosforilación está catalizada por la proteína fosfatasa cdc25 (Norbury y Nurse, 1992).

La actividad del MPF puede ser determinada mediante el análisis de la actividad de la histona H1 quinasa intrínseca a esta proteína. En el bovino y el porcino, la actividad de esta histona H1 quinasa se ve incrementada a las 6 y 20 horas de haber comenzado la maduración, respectivamente, llegando a su primer pico de actividad durante la MI, para posteriormente decrecer durante la anafase I. La actividad de la enzima muestra un segundo pico máximo durante el estadio de MII (Christmann *et al.*, 1994; Fissore *et al.*, 1996).

Existen discrepancias respecto al papel que juega la proteína $p34^{cdc2}$ en la condensación de la cromatina. Por un lado, Kubelka *et al*. (1995) sostienen que la condensación de la cromatina, en ovocitos porcinos, ocurre independientemente de la activación del MPF. Por otro lado, Tatemoto y Terada (1998) sugieren que la $p34^{cdc2}$ ejerce una profunda influencia en la condensación de la cromatina en ovocitos bovinos mientras que Kubelka *et al.* (2000) concluyen que la condensación de la cromatina se da sin que exista la activación del MPF.

La MAPK, una quinasa serina/treonina, es uno de los principales factores que regulan la maduración ovocitaria (Dunphy *et al.*, 1988; Shibuya *et al.*, 1992; Sun *et al.*, 1999b). Esta enzima se activa a través de señales extracelulares específicas y mediante

diferentes vías de transducción (factores de crecimiento y neurotransmisores). La MAPK es activada por la MAPK-quinasa (MAPKK) la cual es, a su vez, fosforilada por varias quinasas aunque, en los ovocitos, el producto del proto-oncogen c-mos es probablemente el que juega el principal papel (Sagata *et al.*, 1989; Nebreda y Hunt, 1993). La activación del MPF y la MAPK, los cuales comparten sustratos incluyendo proteínas implicadas en la formación de la membrana nuclear, condensación de la cromatina y formación del huso (Peter *et al.*, 1992; Verlhac *et al.*, 1994), provocan el reinicio, la progresión y el bloqueo de la meiosis en MII. Una actuación inapropiada o la presencia de bajos niveles de estas quinasas puede afectar negativamente a la capacidad de desarrollo de los ovocitos.

En los ovocitos de ratón y rata (Sun *et al.*, 1999b), la MAPK se activa después de la ruptura de la vesícula germinal y por tanto, coincide con el inicio de la meiosis. También se ha observado que la inhibición del reinicio de la meiosis por AMPc o la proteína quinasa C (PKC) ocurre simultáneamente con la inhibición de la MAPK (Sun *et al.*, 1999a; Sun *et al.*, 1999b; Lu *et al.*, 2001). Algunos estudios muestran que, para que se produzca la ruptura de la vesícula germinal, el citoplasma del ovocito debe tener la habilidad de fosforilar la MAPK (Sun *et al.*, 1999b; Lu *et al.*, 2001).

2.1.2. Inhibición del reinicio de la meiosis.

In vivo, los ovocitos llevan a cabo la maduración citoplasmática durante el crecimiento folicular, periodo caracterizado por una gran síntesis de RNAm y de proteínas necesarias para el reinicio de la meiosis y primeros estadios posteriores a la fecundación (revisado por Wassarman, 1988).

Por el contrario, en los sistemas *in vitro*, el reinicio de la meiosis es inducido espontáneamente por la transferencia de ovocitos meióticamente competentes a medios de cultivo, retirándolos así de su ambiente folicular (Pincus y Enzmann, 1935; Staigmiller y Moor, 1984). Si bien estos ovocitos son capaces de completar la meiosis, no son capaces de realizar la maduración citoplasmática de manera adecuada, lo cual se verá reflejado en su menor capacidad de fecundación y desarrollo embrionario, debido a que probablemente se ha privado a estos ovocitos de factores que se producen en etapas tardías del crecimiento folicular.

Por ello, se ha hipotetizado que si los ovocitos son cultivados *in vitro* antes de su maduración en condiciones que mantengan el bloqueo meiótico en vesícula germinal, éstos pueden tener la oportunidad de madurar citoplasmáticamente y adquirir así, una mayor capacidad para el desarrollo embrionario (Fouladi Nashta *et al.*, 1998).

En un intento para mantener el bloqueo meiótico de los ovocitos se han probado diferentes inhibidores fisiológicos y farmacológicos. Entre los inhibidores fisiológicos citar el fluido folicular (Sirard y First, 1988), las células tecales (Richard y Sirard, 1996b; 1996a) y también las células del *cúmulus* (Petr *et al.*, 1989).

El bloqueo meiótico también puede ser provocado usando diferentes inhibidores farmacológicos como: la ciclohexamida, un inhibidor de la síntesis proteica (Lonergan *et al.*, 1997; Saeki *et al.*, 1997; Le Beux *et al.*, 2003), la 6-dimetilaminopurina, un inhibidor no específico de la fosforilación (Avery *et al.*, 1998), la butirolactona I (BL-I), un compuesto natural que inhibe las quinasas dependientes de ciclinas y que también tiene un efecto inhibitorio sobre otras proteínas quinasas como la MAPK (Kitagawa *et al.*, 1993; Motlik *et al.*, 1998; Kubelka *et al.*, 2000; Lonergan *et al.*, 2000) y la roscovitina (ROS), una purina conocida por inhibir de manera específica la actividad del MPF en numerosos sistemas celulares (Mermillod *et al.*, 2000).

Sin embargo, diferentes estudios han señalado que durante la inhibición del reinicio de la meiosis, tiene lugar algún tipo de progresión meiótica (Fair *et al.*, 2002; Lonergan *et al.*, 2003). De hecho, Vigneron *et al.* (2004) demostraron que algunos eventos relacionados con el reinicio de la meiosis (modificación de la síntesis y fosforilación de algunas proteínas) no son inhibidos en presencia de roscovitina y que la progresión meiótica en ovocitos tratados con roscovitina es más rápida.

La roscovitina ha sido usada en ovocitos de diferentes especies para detener la progresión meiótica: en el bovino, Faerge *et al.* (2001) obtuvieron un 81% de bloqueo meiótico usando 25 µM; Mermillod *et al.* (2000) obtuvieron un 88% de bloqueo y un 36% de blastocistos con la misma dosis; en el equino, Franz *et al.* (2003) observaron un 84% de bloqueo meiótico y un 71% de división embrionaria utilizando una concentración de 66 µM; en el porcino, Ju *et al.* (2003) consiguieron un 83% de

bloqueo meiótico y un 51% de reinicio normal de la meiosis utilizando una concentración de 80 μM; Le Beux *et al.* (2003) obtuvieron un 72 % de bloqueo de la meiosis y un 30 % de reinicio normal de la meiosis utilizando 50 μM y Krischek y Meineke (2001), utilizando 50 μM, observaron un 74% de inhibición de la meiosis y un 76.2% de los ovocitos mostraron condensación de la cromatina a las 28 horas de maduración sin el inhibidor; en el canino, Songsasen *et al.* (2003) consiguieron un 60% de bloqueo de la meiosis utilizando 25 μM.

Donnay *et al.* (2004) observaron que la premaduración de ovocitos de ternera con una combinación de butirolactona y roscovitina (12,5 μM ROS + 6,25μM BL-I) o únicamente con roscovitina (25 μM ROS) evitó el reinicio de la meiosis y su efecto fue reversible después de una maduración *in vitro* durante 17 o 24 horas. Sin embargo, y a diferencia de Mermillod *et al* (2000), evidenciaron una disminución significativa del porcentaje de blastocistos obtenidos después de la fecundación.

Varios autores observaron que la utilización de sustancias que inhiben la meiosis puede causar modificaciones en las estructuras ovocitarias. Faerge *et al.* (2001) observaron que la roscovitina, la butirolactona, el 6-DMAP y la ciclohexamida producen variaciones morfológicas en la membrana nuclear, en la localización de la cromatina en relación con el nucleolo y en la presencia de diferentes poblaciones de gránulos intranucleares. Por otra parte, Ju *et al.* (2003) evidenciaron que entre el 15 – 21% de los ovocitos tratados con roscovitina mostraban una MII anormal con placas metafásicas aberrantes y/o formación aberrante de los microtúbulos citoplasmáticos.

A nivel ultraestructural, Lonergan *et al.* (2003) describieron que la incubación de ovocitos bovinos con BL-1 o ROS alteraba la integridad de las células del *cúmulus* que rodean al ovocito, lo cual afectaba a su expansión durante la maduración *in vitro*. Ya en el citoplasma, observaron que la ROS causaba el hinchamiento de las crestas mitocondriales, mientras que la BL-1 provocaba el aumento de poblaciones de mitocondrias pleomórficas así como el de mitocondrias con matrices lucen. La utilización de cualquiera de los inhibidores causó la degeneración de los gránulos corticales reduciendo su población. Por otro lado, a nivel de núcleo, ambos tratamientos inhibitorios provocaron un enrollamiento de la membrana nuclear y aquellos ovocitos

tratados con ROS presentaron estructuras aberrantes dentro del nucleoplasma (Lonergan *et al.*, 2003).

2.2. Principios generales de crioconservación.

La crioconservación de células junto con su almacenamiento a muy bajas temperaturas es deseable tanto por razones biológicas como por razones comerciales. Al conservar células a temperaturas extremadamente bajas (-196 ºC) es posible detener por completo la actividad enzimática, la respiración celular, el metabolismo, el crecimiento, la multiplicación, etc., es decir, es posible mantener células durante un largo período de tiempo sin afectar a su viabilidad ni causar cambios genéticos (Schneider y Mazur, 1984). No obstante, la mayoría de las células mamíferas mueren cuando se exponen a bajas temperaturas, a menos que previamente hayan sido expuestas a una solución que las proteja y a rangos de enfriamiento y calentamiento específicos (revisado por Shaw *et al.*, 2000).

La crioconservación de gametos y embriones implica el contacto inicial de éstos con soluciones crioprotectoras, su posterior congelación y finalmente su almacenamiento a temperaturas bajo cero. Una vez descongelados, se procede a la dilución y posterior eliminación de los crioprotectores para luego ser transferidos a un ambiente fisiológicamente adecuado que permita a dichas células desarrollarse adecuadamente. Estos pasos representan un gran estrés (estrés mecánico, termal y/o químico) para las células al cual deberán ser capaces de sobrevivir.

Contrariamente a lo que comúnmente se cree, el mayor desafío que las células deben soportar durante la crioconservación no es el causado por el almacenamiento a bajas temperaturas, sino la letalidad de una zona intermedia de temperatura (+15 a -5 ºC), intervalo por el cual las células deben pasar dos veces (una durante la congelación y otra durante la descongelación) (Mazur, 1963).

Los protocolos de crioconservación han sido clasificados como “lentos o rápidos” de acuerdo con la velocidad de enfriamiento y el tipo y concentración de los crioprotectores usados. Sin embargo, los principios y objetivos de la crioconservación son aplicables a ambos: 1. Proteger de los efectos del enfriamiento y congelación, 2.

Evitar la formación de hielo intracelular y 3. Proteger de los efectos tóxicos de los crioprotectores tanto a temperaturas bajas como altas (revisado por Parks y Ruffing, 1992; Critser *et al.*, 1997; Paynter *et al.*, 1999).

2.2.1. Congelación lenta.

La congelación lenta es un técnica de crioconservación en la que existe un "equilibrio" entre la velocidad de enfriamiento, la velocidad de deshidratación y la velocidad de formación de núcleos de hielo. El objetivo principal de este tipo de crioconservación es el de controlar la velocidad de enfriamiento de tal forma que a medida que descienda la temperatura se produzca la penetración del crioprotector al interior de la célula produciéndose un equilibrio osmótico y disminuyendo la probabilidad de formación de cristales de hielo intracelulares.

Para prevenir la formación de hielo intracelular o minimizar el daño que éste pueda causar, todos los protocolos de congelación están destinados a deshidratar las células. En el caso de los protocolos de congelación lenta, este proceso se consigue colocando a las células en una solución que contiene entre un 10 y un 11% (v/v) de crioprotector (aproximadamente 1.5M). A continuación, la temperatura disminuye y se provoca la formación de hielo (*ice seeding*) dentro de esta solución. A medida que los cristales de hielo crecen, el agua de la solución pasa de líquido a sólido y la concentración extracelular de solutos incrementa provocando la salida de agua de la célula. Cuanto más baja es la temperatura, más cantidad de agua puede convertirse en hielo, pero la capacidad de la célula para eliminar el agua intracelular también disminuye a medida que la temperatura disminuye. Por lo tanto, el éxito de un protocolo de congelación lenta se basa en alcanzar el equilibrio entre la velocidad a la que el agua abandona la célula y la velocidad con que este agua se convierte en hielo.

Cuando una célula es enfriada a -5 ºC, tanto la célula como el medio que la rodea se mantienen en un estado superfrío (*supercooled*). Entre -5 y -10 ºC empieza la formación de núcleos de hielo (ya sea espontáneamente o inducidos mediante *seeding*) en el medio externo, mientras que el medio intracelular se mantiene descongelado. Este agua extracelular superfría posee un alto potencial químico y provoca el eflujo de agua desde dentro de la célula hacia afuera, agua que captan los núcleos de hielo extracelular

(revisado por Gao *et al.*, 1997). La célula, al perder agua, se deshidrata y, consecuentemente, se produce la penetración de los crioprotectores para mantener el equilibrio osmótico. Una vez se alcanza la temperatura de formación de hielo (normalmente entre -5 y -9ºC), la temperatura debe descender hasta -33ºC / -40ºC, temperatura a la cual las células pueden ser ya sumergidas directamente en nitrógeno líquido. Las células congeladas según esta metodología deberán descongelarse según protocolos de descongelación rápida (revisado por Shaw *et al.*, 2000).

La velocidad de enfriamiento para esta técnica va de 0,2 a 0,3 ºC/minuto. Sin embargo, para lograr una correcta velocidad de enfriamiento, se necesitan equipos costosos que se encargan, en todo momento, de controlar la tasa de enfriamiento de manera automática.

Si una célula se enfría a velocidades de enfriamiento demasiado rápidas el agua no tiene tiempo para mantener el equilibrio osmótico, es decir, el agua intracelular no tiene tiempo suficiente para salir y formar los núcleos de hielo fuera de la célula y, por tanto, se forman núcleos de hielo intracelular que lesionaran la célula (Mazur, 1990). Por el contrario, si la tasa de enfriamiento celular es demasiado lenta, el eflujo de agua de la célula es tan lento que las células están expuestas durante demasiado tiempo a una elevada concentración de sales y otros solutos. Esta situación puede reducir la supervivencia de dichas células ya que una elevada concentración de sales y solutos puede provocar cambios en el pH intracelular, peroxidaciones lipídicas, etc. (revisado por Gao *et al.*, 1997). Es por esto que se considera que existe un rango de enfriamiento óptimo en el cual existe un equilibrio entre el descenso de la temperatura y la eliminación de agua por parte de la célula.

Son varios los protocolos de congelación lenta utilizados para la congelación de ovocitos. Al congelar ovocitos bovinos en una solución de propilenglicol de 1.5 M, Otoi et al. (1997), Lim et al. (1999) y Mogas et al. (1999) obtuvieron buenos porcentajes de supervivencia de los ovocitos tras el proceso de congelación/descongelación (67.4%, 83% y 48% respectivamente). Sin embargo, el porcentaje de blastocistos obtenidos tras la congelación fue bajo (5%, 3% y 8% respectivamente).

Para intentar mejorar el porcentaje de blastocistos obtenidos por ovocito congelado, diferentes autores han introducido variaciones a los protocolos de congelación lenta. Así se han probado distintos agentes crioprotectores como el propilenglicol, el etilenglicol, el

DMSO y el glicerol (Otoi *et al.*, 1993; Lim *et al.*, 1999; Mogas *et al.*, 1999), distintas velocidades de enfriamiento como por ejemplo 0.3, 0.6 y 0.9ºC/min (Otoi *et al.*, 1994), distintas temperaturas para la inducción del hielo como son -5.5ºC (Otoi *et al.*, 1994) y -9 ºC (Saunders y Parks, 1999), la adición de azúcares (sacarosa) a la solución crioprotectora (Otoi *et al.*, 1995) o la centrifugación previa de los ovocitos (Otoi *et al.*, 1997). Sin embargo, ninguna de las variaciones introducidas en los protocolos de congelación lenta ha permitido elevar los porcentajes de blastocistos obtenidos a niveles superiores al 8% (Mogas *et al.*, 1999). Diferentes autores han sugerido que las soluciones utilizadas durante la congelación lenta podrían ser mejoradas añadiendo EDTA (Mogas *et al.*, 1999), citocalasina (Younis *et al.*, 1997), pequeñas moléculas lipofílicas como el hidroxitolueno butilado (Zeron *et al.*, 1999), proteínas anticongelantes (Arav *et al.*, 1993a) o sales (Stachecki *et al.*, 1998).

2.2.2. Congelación rápida y ultrarrápida.

Esta técnica previene la formación de hielo intracelular mediante la deshidratación de la célula. Para conseguirlo, se expone a la célula a altas concentraciones de un crioprotector permeable y, posteriormente, a un enfriamiento rápido o ultrarrápido.

Los protocolos de congelación rápida y ultrarrápida utilizan altas concentraciones de solutos (crioprotectores y azúcares) que eliminan rápidamente el agua de las células. En estas soluciones, las células se deshidratan rápidamente y se favorece la entrada de los crioprotectores, lo que permite sumergirlas directamente en nitrógeno líquido (congelación ultrarrápida) o vapores de nitrógeno (congelación rápida). Las tasas de congelación conseguidas con las técnicas de congelación ultrarrápidas (11000 a 14000 ºC/minuto) disminuyen drásticamente el daño por enfriamiento, permitiendo usar soluciones crioprotectoras menos concentradas (menos tóxicas) y acortar el tiempo de exposición del ovocito al crioprotector (Martino *et al.*, 1996).

Los protocolos de congelación rápida se dividen en dos subcategorías dependiendo de si existe (congelación rápida o ultrarrápida) o no (vitrificación) formación de hielo en la solución durante la congelación. Si bien la diferenciación entre congelación ultrarrápida y vitrificación no está bien establecido, se debería utilizar el término vitrificación sólo para aquellas técnicas en las que no se forman cristales de hielo durante la congelación y descongelación, ni intracelular ni extracelular. Por el contrario, si se forman aunque

sólo sean trazas de hielo durante estos procesos, el término correcto debería ser congelación ultrarrápida (Shaw *et al.*, 2000).

2.3. Crioconservación de ovocitos.

El ovocito es un tipo celular único que posee un gran tamaño y una baja relación superficie-volumen. Además, está rodeado por la zona pelúcida (ZP) y por varias capas de células del *cúmulus oophorus*. La presencia de estas capas celulares que rodean al ovocito y que juegan un papel importante durante la etapa de crecimiento del ovocito mediante una cooperación metabólica, hacen de este tipo celular una estructura muy difícil de crioconservar (revisado por Massip, 2003). Hasta la actualidad, se han podido crioconservar ovocitos de varias especies animales utilizando diferentes técnicas y crioprotectores: en el conejo, por congelación lenta (Al-Hasani *et al.*, 1989) y rápida (Vincent *et al.*, 1989); en la rata, por congelación lenta (Kasai *et al.*, 1979) y ultrarrápida (Isachenko *et al.*, 2000); en el ratón, por congelación lenta (Stachecki *et al.*, 1998), ultrarrápida (Aono *et al.*, 2003) y vitrificación (Kasai *et al.*, 1990; O´Neil *et al.*, 1997; Chen *et al.*, 2000; Tokieda *et al.*, 2002); en la vaca, por congelación lenta (Lim *et al.*, 1991; Fuku *et al.*, 1992; Otoi *et al.*, 1995), ultrarrápida (Martino *et al.*, 1996; Rho *et al.*, 2002) y vitrificación (Vajta *et al.*, 1998; Hyttel *et al.*, 2000; Vieira *et al.*, 2002); en la yegua, por congelación lenta (Hochi *et al.*, 1994) y por vitrificación (Hurtt *et al.*, 2000); en la cerda, por congelación ultrarrápida (Isachenko *et al.*, 1998) y por vitrificación (Nagashima *et al.*, 1996; Rojas *et al.*, 2004) y en la mujer, por congelación lenta (Boiso *et al.*, 2002) y por vitrificación (Liebermann y Tucker, 2002).

Desafortunadamente, los protocolos desarrollados para una especie en concreto son usualmente muy difíciles de adaptar a otra especie debido a diferencias en el tamaño del ovocito y a su sensibilidad al enfriamiento y a los crioprotectores (revisado por Shaw *et al.*, 2000). El factor más crítico para la crioconservación de ovocitos es su compleja organización subcelular. Los efectos del enfriamiento afectan a los elementos del citoesqueleto como son la placa metafásica o la integridad de los microtúbulos (Shaw *et al.*, 2000) y también puede afectar a los gránulos corticales produciendo el endurecimiento de la zona pelúcida (Parks y Ruffing, 1992).

2.3.1. Efectos del enfriamiento y la congelación sobre las estructuras ovocitarias.

La mayoría de los estudios sobre la hipotermia en ovocitos mamíferos se limitan principalmente a los efectos de ésta sobre la morfología, la citología y la competencia para el desarrollo embrionario de los ovocitos enfriados o crioconservados y, más concretamente, de los ovocitos que se encuentran en estadio de MII (Parks, 1997).

Cuando se somete a un ovocito a temperaturas inferiores a las fisiológicas y estas se elevan nuevamente a 37 ºC, el ovocito sufre una serie de cambios físicos y fisiológicos. No obstante, los mayores daños se producen entre +15 y -5 ºC. Temperaturas entre +30 y 0 ºC pueden comprometer la integridad de la membrana, el metabolismo celular, el citoesqueleto y la capacidad celular de controlar y reparar los daños producidos por los radicales libres (Arav *et al.*, 1996; Parks, 1997). Por otro lado, las temperaturas por debajo de 0 ºC suponen un alto riesgo de formación de hielo intracelular el cual puede provocar un daño irreparable en la célula (Trad *et al.*, 1999).

2.3.1.1. Efectos de la temperatura sobre la placa metafásica.

El cambio más drástico que sufren los ovocitos maduros cuando son sometidos a cambios de temperatura es el desensamblaje de la placa metafásica (Magistrini y Szollosi, 1980). La placa metafásica está compuesta por microtúbulos que contienen subunidades α y β de dímeros de tubulina en equilibrio dinámico entre la forma libre y la polimerizada. Este equilibrio es extremadamente termosensible y tiende al desensamblaje por debajo de la temperatura fisiológica. Los cromosomas se encuentran fuertemente unidos a la placa metafásica y, en ausencia de una envoltura nuclear, se considera que la placa metafásica es una estructura relativamente inestable (revisado por Bernard y Fuller, 1996).

La elevada termosensibilidad de la placa metafásica ha sido observada en ovocitos murinos (Magistrini y Szollosi, 1980; Pickering y Johnson, 1987), humanos (Pickering *et al.*, 1990) y bovinos (Aman y Parks, 1994) no importando el tipo de crioconservación utilizado: congelación lenta (Vincent *et al.*, 1989; Eroglu *et al.*, 1998), ultrarrápida (Aigner *et al.*, 1992) o vitrificación (Chen *et al.*, 2003). Magistrini y Szollosi (1980) observaron que los microtúbulos de la placa metafásica de ovocitos de ratón se

despolimerizaban después de 15 minutos de exposición a 0 ºC y que desaparecían completamente después de 45 – 60 minutos de exposición. Sin embargo, un progresivo calentamiento de los ovocitos hasta la temperatura de 37 ºC provocó la repolimerización de los microtúbulos y la formación de la placa metafásica. Esta habilidad para la reorganización de la placa metafásica fue confirmada por Pickering y Johnson (1987) en ovocitos de la misma especie. Saunders y Parks (1999) estudiaron los cambios provocados en la distribución de los cromosomas, microtúbulos y microfilamentos tras el enfriamiento, el contacto con el crioprotector (1.5 M de etilenglicol) o la congelación de ovocitos bovinos madurados *in vitro*. En los ovocitos que sólo habían sido enfriados a -9ºC durante 12 minutos observaron cambios que incluían dispersión o agrupamiento de cromosomas, despolimerización de los microtúbulos y la alteración de la estructura del huso cromosómico.

Aman y Parks (1994) demostraron que el enfriamiento de ovocitos bovinos madurados *in vitro* a +25 ºC o a +4 ºC daba lugar a una disgregación o disociación parcial o total del huso cromosómico y a la dispersión de algunos cromosomas. El recalentamiento de los ovocitos a +37ºC desde +25ºC restauraba la morfología normal del huso, mientras que los ovocitos que habían sido enfriados a +4ºC y recalentados presentaban husos cromosómicos anormales. Por otro lado, tras someter ovocitos bovinos en estadio de VG a diferentes temperaturas, Wu *et al.* (1999) observaron que temperaturas superiores a 24 ºC no reducen los porcentajes de placas metafásicas normales ni los porcentajes de fecundación y división embrionaria de los ovocitos. Sin embargo, cuando se somete a los ovocitos a temperaturas cercanas a los 4 ºC ni que solo sea por 10 minutos, se reduce drásticamente el porcentaje de husos meióticos normales y los porcentajes de división y fecundación. Entre los daños provocados por la refrigeración sobre la estructura de la placa metafásica se pudo observar desorganización, descondensación y ausencia de los cromosomas. A nivel de microtúbulos, la exposición a bajas temperaturas afectó a la organización microtubular provocada por la desorganización de los microtúbulos o la ausencia de algunos o todos los microtúbulos. También como consecuencia de las bajas temperaturas, Vincent y Jonson (1992) observaron un alineamiento cromosómico anormal y la segregación de cromátidas en ovocitos de conejo.

Diversos autores han demostrado que la congelación lenta (Eroglu *et al.*, 1998; Saunders y Parks, 1999), rápida (Vincent *et al.*, 1989), ultrarrápida (Aigner *et al.*, 1992)

y vitrificación (Chen *et al.*, 2000) causan una severa desorganización o desaparición de las placas metafásicas inmediatamente después de la congelación. En la congelación lenta de ovocitos bovinos en estadio de MII, Saunders y Parks (1999) observaron que ésta causaba la desorganización parcial o total de los cromosomas y/o los microtúbulos, la despolimerización de los microtúbulos y la descondensación de los cromosomas. Similares resultados fueron observados por Boiso *et al.* (2002) en ovocitos humanos madurados *in vitro*, quienes describieron que la congelación lenta puede llegar a producir la desorganización o total desaparición de los microtúbulos, la aparición de cromosomas aberrantes y la descondensación parcial de los mismos.

Sin embargo, la posterior incubación de estos ovocitos a temperatura fisiológica resultó en la recuperación de la estructura de la placa metafásica. Así, varios autores han observado que la recuperación de la estructura de la placa metafásica requiere un período de incubación posterior a la descongelación y que a su vez depende del método de congelación y de la especie animal (Aigner *et al.*, 1992; Gook *et al.*, 1993; Chen *et al.*, 2001). En el caso de los bovinos, Saunders y Parks (1999) observaron que un 64% de los ovocitos congelados recuperaban la estructura de la placa metafásica a los 20 minutos después de ser congelados mediante congelación lenta. Eroglu *et al*. (1998) observaron que los ovocitos de ratón congelados mediante congelación lenta son capaces de recuperar completamente la estructura de la placa metafásica después de 1 hora de incubación. Este período se alargó hasta 2 o 3 horas cuando estos ovocitos fueron congelados por congelación ultrarrápida (Aigner *et al.*, 1992) o vitrificación (Chen *et al.*, 2001). En el caso de ovocitos humanos Gook *et al*. (1993) observaron que un 60% de los ovocitos congelados mediante congelación lenta recuperaban la estructura normal de la placa metafásica después de 1 hora de incubación.

Estos cambios observados en la placa metafásica debidos al proceso de congelación/descongelación provocan cambios funcionales del ovocito durante la fecundación y posterior desarrollo embrionario (Eroglu *et al.*, 1998; Chen *et al.*, 2001), reportándose en estos ovocitos un incremento de las poliploidías después de la fecundación (Glenister *et al.*, 1987; Carroll *et al.*, 1989; Bouquet *et al.*, 1992; Eroglu *et al.*, 1998; Luna *et al.*, 2001).

2.3.1.2. Efectos de la temperatura sobre el citoesqueleto ovocitario.

Los microfilamentos están compuestos por actina polimerizada la cual se encuentra en equilibrio dinámico con la actina libre, de manera similar a los microtúbulos. Mientras que los microtúbulos son importantes para aquellos procesos que involucran la correcta distribución de los cromosomas en la placa metafásica, los microfilamentos son igualmente importantes en diferentes etapas, incluyendo la citoquinesis, la rotación de la placa metafásica, la extrusión del corpúsculo polar y la migración pronuclear (Vincent y Johnson, 1992).

En contraste con los microtúbulos, los microfilamentos que forman el citoesqueleto cortical del ovocito no parecen estar modificados directamente por el enfriamiento (revisado por Parks, 1997). Sin embargo, existe la posibilidad que éstos se vean afectados por las diferentes etapas de la crioconservación (revisado por Bernard y Fuller, 1996).

Varios autores observaron que la congelación causaba cambios en la organización del citoesqueleto de actina en ratón, conejo y vaca (Vincent *et al.*, 1989; George y Johnson, 1993; Saunders y Parks, 1999, respectivamente). En estos ovocitos se pudo observar la desaparición de la banda de actina por despolimerización y por segmentación (Vincent *et al.*, 1989). Cuando los ovocitos de ratón se evaluaron inmediatamente después de la congelación, Eroglu *et al.* (1998) observaron alteraciones en el citoesqueleto tales como la disrupción de la red de microfilamentos y la aparición de núcleos de microtúbulos en el citoplasma. Sin embargo, estos cambios no fueron dramáticos y en su mayoría fueron reversibles después de un período de incubación posterior a la descongelación. De hecho, Saunders y Parks (1999) observaron un bajo porcentaje de alteraciones en la banda de actina después de congelar ovocitos bovinos por congelación lenta.

La desestructuración del citoesqueleto puede ser debida a los cambios de forma y volumen que acompañan a los procesos de congelación/descongelación, cambios que pueden provocar la liberación prematura de los gránulos corticales y el endurecimiento de la zona pelúcida (revisado por Vincent y Johnson, 1992). Por este motivo, aunque la distribución de los microfilamentos del ovocito pueda recomponerse tras la congelación o el contacto con los agentes crioprotectores, pueden haberse dado ya alteraciones irreversibles de otros

componentes celulares como la zona pelúcida, membrana plasmática o las mitocondrias, debido a la asociación existente entre los microfilamentos y estas estructuras.

2.3.1.3 Efectos de la temperatura sobre otras estructuras ovocitarias.

La exocitosis de los gránulos corticales es una respuesta normal a la unión del espermatozoide fecundante con el oolema. Las proteasas acrosomales son eliminadas al espacio perivitelino y modifican los componentes glucoproteicos de la zona pelúcida, responsable de la unión y penetración del espermatozoide, previniendo posteriores fecundaciones y bloqueando así la poliespermia (Webb *et al.*, 1986; Wassarman y Albertini, 1994). La migración y la liberación de los gránulos corticales involucra un correcto funcionamiento del citoesqueleto y de la organización de la membrana plasmática (revisado por Bernard y Fuller, 1996).

El enfriamiento de los ovocitos de ratón a 4 ºC durante 5 minutos alteró dramáticamente la zona pelúcida reduciendo el porcentaje de fecundación (Vincent *et al.*, 1990). Estos cambios estarían asociados a una exocitosis prematura de los gránulos corticales, lo que provocaría el endurecimiento de la zona pelúcida (Carroll *et al.*, 1990; Vincent y Johnson, 1992). Fuku *et al.* (1995) observaron pequeños cambios en la zona pelúcida como la presencia de irregularidades, fracturas y cambios en la densidad electrónica cuando vitrificaron ovocitos bovinos en pajuelas de 0.25 ml. Los autores también asociaron estos cambios a la prematura exocitosis de los gránulos corticales. Hyttel *et al.* (2000) confirmaron este hecho al detectar por microscopía electrónica la ausencia de la típica banda de gránulos corticales por debajo de la membrana plasmática al vitrificar ovocitos bovinos mediante la técnica *Open pulled straw* (OPS). Además, estos autores observaron clusters de gránulos corticales en distintas etapas de degeneración (Hyttel *et al.*, 2000).

La congelación también causa profundos cambios en otras estructuras ovocitarias. Fuku *et al.* (1995) observaron profundas modificaciones ultraestructurales tras la vitrificación de ovocitos bovinos. Estos autores evidenciaron la ruptura de las uniones gap o la ausencia de microvellocidades. A nivel de mictocondrias, las anormalidades se centraban en la presencia de extensas áreas vacuolizadas, la desaparición de la mayoría de las crestas mitocondriales y alteraciones en la membrana mitocondrial (Fuku *et al.*,

1995), así como también una marcada disminución en el número de las mitocondrias (Rho *et al.*, 2002). También evidenciaron la presencia de cambios en la zona pelúcida en los ovocitos madurados *in vitro,* debidos a la vitrificación, los cuales fueron asociados a una disminución en el número de gránulos corticales en el ooplasma debido a que la congelación causa una liberación prematura de éstos.

A nivel de DNA, Men *et al*. (2003) observaron que un elevado porcentaje de ovocitos que microscópicamente no presentaban daño morfológico presentaban alteraciones a nivel de DNA tras la congelación lenta o vitrificación de ovocitos bovinos madurados *in vitro*.

2.4. Vitrificación.

La vitrificación se define como la transición de las soluciones acuosas de un estado líquido a un estado vítreo sólido sin la formación de cristales, es decir, que debido al rápido descenso de temperatura, la "viscosidad" de la muestra aumenta hasta un punto en que las moléculas se inmovilizan. De esta forma, se encuentran en un estado sólido aunque su estructura molecular sea la de un líquido extremadamente viscoso (estado vítreo) (Critser *et al.*, 1997). Este aumento extremo de la viscosidad requiere velocidades de enfriamiento muy rápidas (superiores a 2500 ºC/min) o elevadas concentraciones de crioprotectores (de 5 a 7 M) (revisado por Shaw *et al.*, 2000; Vajta, 2000).

La vitrificación presenta numerosas ventajas como la total eliminación de la formación de hielo o la disminución del daño causado por el enfriamiento, puesto que atraviesa el rango de temperatura de +15 a –5 ºC a velocidades de enfriamiento muy rápidas (Dobrinsky, 1996; Martino *et al.*, 1996; Isachenko *et al.*, 1998; Zeron *et al.*, 1999). Otra gran ventaja de esta técnica es que no requiere de equipos de congelación caros o sofisticados y puede ser realizada de manera muy sencilla.

La consecuencia negativa de esta estrategia radica en el incremento de las probabilidades de lesionar las células debido al choque osmótico y a la toxicidad de los crioprotectores. Sin embargo, se han aplicado diferentes protocolos para intentar disminuir estos efectos negativos, como el uso de crioprotectores menos tóxicos o la

combinación de crioprotectores (disminuyendo la toxicidad individual de cada uno, pero manteniendo las propiedades osmóticas y crioprotectoras), la utilización de crioprotectores por etapas (*stepwise*) y/o la utilización de soluciones concentradas preenfriadas (revisado por Vajta, 2000).

2.4.1. Factores que influyen en la vitrificación.

2.4.1.1 Velocidades de congelación y descongelación.

Uno de los parámetros más importantes para llevar a cabo la vitrificación con éxito es la velocidad de enfriamiento. Existe una velocidad de enfriamiento límite, que es la velocidad límite biológica que puede soportar una célula a una concentración de crioprotector dada. Por tanto, el equilibrio entre la máxima velocidad de congelación y la concentración del crioprotector será de mucha importancia para conseguir resultados óptimos (Critser *et al.*, 1997; Liebermann *et al.*, 2002a). La principal estrategia de la vitrificación es pasar el rango de temperatura crítica lo más rápido posible para así disminuir el riesgo de daño celular y llevar a la célula hasta la temperatura de -196 ºC. Para el soporte OPS, las velocidades de enfriamiento que se consiguen son cercanas a los 20000 ºC/minuto.

Un tejido que ha sido vitrificado y almacenado a temperaturas de nitrógeno líquido deberá ser descongelado a temperatura ambiente antes de continuar con su cultivo y/o desarrollo. Los protocolos de desvitrificación deberán tratar de disminuir o eliminar la probabilidad de recristalización mediante una adecuada transferencia térmica (revisado por Critser *et al.*, 1997). Para descongelar una muestra vitrificada, se sumerge el soporte de vitrificación en un medio a temperatura fisiológica (37 ºC). De esta forma, se obtienen velocidades de calentamiento entre 3000 y 8000 ºC/minuto, dependiendo del soporte utilizado para la vitrificación (Hochi *et al.*, 2001).

2.4.1.2 Crioprotectores y aditivos crioprotectores.

Los crioprotectores o CPA (*Cryoprotector Agent*) se clasifican, desde el punto de vista farmacológico, como drogas de acción inespecífica que permiten a las células sobrevivir a la congelación del agua a través de diferentes mecanismos, es decir, no consiguen su

efecto actuando directamente sobre receptores, enzimas o genes específicos. Entre los diferentes CPA, citar los alcoholes (incluyendo los glicoles), las aminas (incluyendo las amidas), los azúcares, las sales inorgánicas y las macromoléculas (incluyendo proteínas y polisacáridos). A pesar de su variada naturaleza química, todos los CPA son solubles en medios acuosos y tienen capacidad de formar puentes de hidrógeno (revisado por Karrow, 1997). Las drogas no específicas, al igual que las específicas, también presentan efectos adversos sumados a los efectos deseados.

Los crioprotectores tratan de evitar los daños causados por la congelación. Para conseguir este objetivo, actúan básicamente sobre dos aspectos de la congelación (Karrow, 1997):

1. Sobre la formación de cristales de hielo, puesto que inicialmente disminuyen la temperatura a la cual se forman los núcleos de hielo (de 0 ºC a -4/-5 ºC), es decir, favorecen la formación de agua "*superfría*" sin que se inicie la formación de cristales de hielo.
2. Sobre la deshidratación de la célula, debido a su elevada osmolaridad. Esta deshidratación también previene la formación de cristales de hielo intracelulares.

2.4.1.2.1. Crioprotectores permeables.

Los crioprotectores pueden ser clasificados en permeables o impermeables, según tengan o no capacidad para atravesar la membrana plasmática. Los crioprotectores permeables, debido a que tienen un peso molecular bajo, son capaces de atravesar la membrana plasmática de forma activa o pasiva. Entre éstos, se encuentran los alcoholes como el glicerol, el etilenglicol, el propilenglicol o el sorbitol (Shaw *et al.*, 1995; Shaw *et al.*, 2000) y las aminas como la acetamida, la betaína, la formamida, la glutamina, la lisina o la taurina (Karrow, 1997). De todos, el etilenglicol es el CPA permeable más ampliamente usado para la vitrificación de embriones y ovocitos debido a su baja toxicidad celular (Kasai *et al.*, 1992; Ali y Shelton, 1993; Kasai, 1996) y a su rápida capacidad de difusión a través de la membrana plasmática (Emiliani *et al.*, 2000).

La eficacia del etilenglicol como crioprotector ha sido ampliamente descrita y se han realizado diferentes estudios para tratar de determinar las concentraciones óptimas y los tiempos de exposición. Hotamisligil *et al.* (1996) vitrificaron ovocitos de ratón con una

concentración 6 M de etilenglicol y 0,5 M de sacarosa no encontrando diferencias significativas entre los ovocitos vitrificados y los control en el porcentaje de blastocistos obtenidos. Otoi *et al.* (1997) observaron un mayor porcentaje de supervivencia y de blastocistos vitrificando ovocitos bovinos con un 40% de etilenglicol y una dilución en tres pasos. Isachenko *et al.* (2001) obtuvieron un 22% de ovocitos en estadio de MII después de vitrificar ovocitos porcinos en estadio de vesícula germinal utilizando un 40% de etilenglicol.

2.4.1.2.2. Crioprotectores no permeables.

Los crioprotectores no permeables no son capaces de atravesar la membrana plasmática debido a su elevado peso molecular y a su compleja estructura. Además, no presentan efecto crioprotector por si solos. Su principal función crioprotectora es la de elevar la presión osmótica, disminuyendo, de esta forma, la cantidad requerida de crioprotector permeable y su toxicidad, y favoreciendo así la deshidratación de la célula (Shaw *et al.*, 1997). Dentro de este grupo se encuentran los azúcares tales como la glucosa, la fructosa, la sacarosa, la trealosa y la lactosa. Estas macromoléculas son capaces de extraer el agua libre intracelular utilizando la diferencia de presión osmótica pero sin penetrar en la célula. Diferentes estudios han evidenciado que estas moléculas son capaces de encapsular al ovocito o embrión en una matriz viscosa previniendo la cristalización intracelular durante la descongelación (Kuleshova *et al.*, 1999) y actuando como tampón osmótico al reducir el choque osmótico que podría resultar de la dilución del crioprotector (Liebermann *et al.*, 2002a). También se ha observado que la adición de azúcares a los medios de crioconservación favorece la estabilidad de la membrana plasmática durante los procesos de vitrificación y descongelación (Crowe *et al.*, 1983).

Las soluciones crioprotectoras también se pueden suplementar con macromoléculas como la albúmina o los polímeros sintéticos (ficoll, dextrano, polivinilpirrolidona, polietilenglicol). Recientes estudios avalan los efectos potencialmente beneficiosos de adicionar estas macromoléculas a la solución de vitrificación.(O´Neil *et al.*, 1997) ya que aumentan la viscosidad de la solución de vitrificación y reducen la toxicidad de las soluciones de vitrificación al reducir las concentraciones de crioprotectores requeridas (Liebermann y Tucker, 2002). Dichos polímeros también pueden construir una matriz viscosa que encapsularía los ovocitos/embriones y ayudaría a prevenir la cristalización

durante el proceso de congelación y descongelación (Kasai *et al.*, 1990; Kuleshova *et al.*, 2001). O'Neil *et al.* (1997) observaron que la adición de polietilenglicol a la solución de vitrificación incrementaba significativamente la supervivencia de los ovocitos de ratón.

2.4.1.3. Estadios de maduración.

Otro de los factores a considerar en el proceso de vitrificación son los estadios de maduración meiótica de los ovocitos, los cuales pueden influir en su capacidad para sobrevivir a la criopreservación (revisado por Shaw *et al.*, 2000). Desde el punto de vista criobiológico existen importantes diferencias entre los ovocitos inmaduros que se encuentran en los folículos primordiales, los ovocitos en vesícula germinal y aquellos en MII (Critser *et al.*, 1997).

La congelación de ovocitos en estadio de VG podría presentar ventajas ya que los microtúbulos aún no están organizados en forma de huso y el material genético está dentro del núcleo protegido por la membrana nuclear (revisado por Arav *et al.*, 1993b). Sin embargo, diferentes estudios revelan que los ovocitos en estadio de VG son más sensibles a los daños por congelación que los ovocitos en otros estadios nucleares (Parks y Ruffing, 1992; Otoi *et al.*, 1995). Así, a pesar de que ya se han obtenido blastocistos (Kuchenmeister y Kuwayama, 1997; Le Gal y Massip, 1999), gestaciones (Otoi *et al.*, 1995)) y terneros (Suzuki *et al.*, 1996; Kubota *et al.*, 1998; Vieira *et al.*, 2002) a partir de la congelación de ovocitos bovinos inmaduros, los índices de supervivencia de estos ovocitos y el porcentaje de blastocistos obtenidos es muy bajo. También en el porcino Didion *et al* (1990), Isachenko *et al.* (2001) y Rojas *et al.* (2004) obtuvieron bajos porcentajes de supervivencia al vitrificar ovocitos inmaduros de cerdo.

Diferentes estudios han determinado que el estadio de vesícula germinal es más sensible a la criopreservación que otros estadios nucleares debido a que el proceso de congelación dañaría las membranas plasmáticas (Zeron *et al.*, 1999) cuya integridad es necesaria para la comunicación intercelular entre el ovocito y las células del *cúmulus*, perjudicando así su posterior maduración (Parks y Ruffing, 1992; Otoi *et al.*, 1995; Fuku *et al.*, 1995; Hochi *et al.*, 1998). Por otra parte, Allworth y Albertini (1993) observaron cambios en los componentes del citoesqueleto entre los ovocitos bovinos en

estadio de VG o MII. Así, mientras que en los ovocitos en estadio de VG, los microtúbulos y los microfilamentos aparecían rígidos y rectos, estos elementos se mostraban ondulantes y flexibles en el estadio de MII . Park *et al.* (1997) observaron un mayor número de placas metafásicas anormales en ovocitos humanos congelados en VG y posteriormente madurados *in vitro*.

Un intento por mejorar los porcentajes de supervivencia y desarrollo embrionario de los ovocitos congelados en VG consistiría en premadurar al ovocito antes de ser crioconservado. De esta forma, el ovocito podría ser congelado en estadio de VG, estadio donde las estructuras cromosómicas y microtubulares estarían más protegidas mientras que su competencia citoplasmática para desarrollarse tras la congelación se vería incrementada. Esta premaduración podría conseguirse tras una cultivo previo de ovocitos inmaduros con inhibidores de las quinasas dependientes de ciclinas como pueden ser la roscovitina o la butirolactona (Mermillod *et al.*, 2000; Lonergan *et al.*, 2003).

El índice más alto de blastocistos obtenidos tras un proceso de vitrificación ha sido a partir de ovocitos bovinos criopreservados en estadio de MII utilizando la técnica OPS (25% blastocistos: Vajta *et al.*, 1998). Sin embargo, Le Gal y Massip (1999), en bovino, obtuvieron peores resultados utilizando también la OPS como soporte (3.3% y 1.8% en VG y MII respectivamente). Martino *et al.* (1996) obtuvieron un 15% de blastocistos utilizando gradillas de microscopía electrónica como soporte; Rho *et al.* (2002) obtuvieron un 7,7 y 8.4% de blastocisitos después de vitrificar ovocitos en MII con *open pulled glass* y gradillas de microscopía electrónica, respectivamente. Como posibles causas del bajo porcentaje de blastocistos obtenidos en los estudios de vitrificación de ovocitos en estadio de MII, citar el daño estructural irreversible provocado por la criopreservación en la membrana del ovocito (Arav y Zeron, 1997), en la distribución de los gránulos corticales (Fuku *et al.*, 1995; Hyttel *et al.*, 2000) o en la desorganización del huso con las consecuentes alteraciones de los cromosomas en los ovocitos en MII (Aman y Parks, 1994; Hochi *et al.*, 1998; Saunders y Parks, 1999; Chen *et al.*, 2001; Boiso *et al.*, 2002), los cuales pueden causar aneuploidía o poliploidía (Sathananthan *et al.*, 1987; Eroglu *et al.*, 1998).

Existen algunos trabajos en los que se ha planteado la posibilidad de congelar en estadios intermedios de desarrollo, como por ejemplo, el estadio de VGBD (Men *et al.*, 2002) o en el periodo entre MI y MII (Hochi *et al.*, 1998; Le Gal y Massip, 1999) con un relativo éxito. Men *et al* (2002), tras vitrificar ovocitos bovinos en GVBD o MII, obtuvieron porcentajes de división similares en ambos estadios. Sin embargo, el porcentaje de blastocistos obtenido fue significativamente mayor para aquellos ovocitos vitrificados en estadio de MII respecto a los de GVBD.

Luna *et al.* (2001) observaron que el estadio de maduración influye sobre el porcentaje de ovocitos bovinos diploides obtenidos después de la vitrificación, obteniendo mejores resultados al vitrificar ovocitos en estadio de MII. Le Gal y Massip (1999) no observaron diferencias significativas en el porcentaje de división celular a las 48 horas post-inseminación o en el porcentaje de blastocistos obtenidos tras vitrificar ovocitos bovinos a las 0, 17 y 24 horas del inicio de la maduración. En el porcino, Rojas *et al.* (2004) obtuvieron un 10.4% de embriones en estadio de 2 a 4 células tras vitrificar ovocitos en estadio de MII mientras que cuando vitrificaron ovocitos en estadio de VG, ninguno de los ovocitos se desarrolló tras su FIV.

2.4.1.4 Soportes para vitrificación.

Para conseguir que la velocidad de enfriamiento sea lo suficientemente rápida para evitar la formación de cristales de hielo, es imprescindible que el volumen a vitrificar sea lo más pequeño posible. Cuando las células son sumergidas directamente en el nitrógeno líquido (LN_2) éste se calienta por el contacto con la célula, produciéndose una gran una capa de vapor que rodea a la célula, aislando la muestra y evitando así, el intercambio térmico entre la muestra y el LN_2. Dicho proceso provoca la disminución de la velocidad de enfriamiento de la célula. Para prevenir esta disminución en la velocidad de enfriamiento, la muestra a vitrificar deberá estar contenida en el menor volumen posible para así tener una mayor superficie de contacto con el LN_2.

Las diferentes técnicas de vitrificación utilizan una gran variedad de soportes para minimizar el volumen a vitrificar. Entre estas podemos mencionar a las pajuelas *open pulled straws* (Vajta *et al.*, 1998; Vieira *et al.*, 2002), las pajuelas *closed pulled straw* (CPS) (Chen *et al.*, 2001), las *flexipet-denuding pipette* (FDP) (Liebermann *et al.*,

2002b), las gradillas de microscopia electrónica (Martino *et al.*, 1996; Chen *et al.*, 2003), el sistema *hemistraw*, el *nylon mesh* (Matsumoto *et al.*, 2001), los *cryoloop* (Mavrides y Morroll, 2002), las microgotas (Begin *et al.*, 2003) y las pajuelas de 0,25 mL. (Otoi *et al.*, 1998; Chen *et al.*, 2001). La velocidad de enfriamiento que se consigue al vitrificar ovocitos utilizando rejillas de microscopía electrónica es de 11000 a 14000 ºC/minuto (Martino *et al.*, 1996). Con la técnica OPS se logra una tasa de enfriamiento de aproximadamente 20000 ºC/minuto (Vajta, 2000). La vitrificación con la técnica OPS ha demostrado ser una de las más eficientes para la crioconservación de embriones bovinos en estadios de precompactación y preimplantacionales (Vajta *et al.*, 1998). Al vitrificar ovocitos bovinos, Martino *et al.* (1996) y Vajta *et al.* (1998) obtuvieron un 12% y un 25% de blastocistos respectivamente, vitrificando ovocitos en gradillas de microscopía electrónica y OPS, respectivamente.

2.5. Análisis comparativo entre ovocitos de animales prepúberes y adultos.

La posibilidad de obtener embriones viables procedentes de animales prepúberes podría permitir la disminución del intervalo generacional y, de esta forma, incrementar la presión genética en los programas de mejora genética. Sin embargo, se ha observado que la utilización de animales donantes juveniles o prepúberes en los programas de transferencia embrionaria bovina no ha dado los resultados esperados, obteniéndose una baja tasa de fecundación y un pobre potencial de desarrollo embrionario *in vitro* (revisado por Gandolfi *et al.*, 1998).

Hasta la fecha, se ha conseguido en varias ocasiones la preñez y el nacimiento de terneros procedentes de ovocitos de animales prepúberes fecundados *in vitro* (Armstrong *et al.*, 1992; Revel *et al.*, 1995). Sin embargo, estos ovocitos muestran una baja eficiencia comparados con los resultados obtenidos a partir de ovocitos de animales adultos. Así, Taneja *et al.* (2000) demostraron que hasta que la ternera no cumple 11 meses, los ovocitos no muestran un potencial de desarrollo similar al de los animales adultos. Esta baja eficiencia se debe en gran medida a las diferencias estructurales y fisiológicas que presentan los ovocitos prepúberes como son la incapacidad para completar la maduración citoplasmática (Salamone *et al.*, 2001), el menor diámetro celular (Armstrong, 2001) y un menor metabolismo energético (Gandolfi *et al.*, 1998), enzimático y proteico (Driancourt *et al.*, 1991).

Las deficiencias en la maduración citoplasmática que presentan los ovocitos procedentes de animales prepúberes pueden expresarse durante la fecundación *in vitro* o en diferentes etapas del desarrollo embrionario. Estas deficiencias juegan un papel importante en la regulación celular, incluyendo la regulación de la meiosis (Balakier y Czolowska, 1977), y pueden expresarse como:

a) Fallo en la penetración y descondensación del espermatozoide.
b) Incapacidad para la formación del pronúcleo masculino.
c) Fallo en el bloqueo de la poliespermia.
d) Incapacidad para llevar adelante la primera división celular.
e) Incapacidad para activar el genoma embrionario.
f) Incapacidad de poder llevar adelante el desarrollo embrionario produciéndose pérdidas embrionarias tanto en etapas preimplantacionales como postimplantacionales (Armstrong, 2001).

Khatir *et al.* (1998) observaron que si bien los ovocitos procedentes de animales prepúberes conseguían realizar la maduración nuclear y las primeras divisiones embrionarias en un porcentaje más o menos cercano al de los ovocitos adultos, éstos no lograban una correcta maduración citoplasmática ya que la tasa de blastocistos era superior en ovocitos procedentes de animales adultos. Este hecho fue confirmado por Mermillod *et al.* (1998) a través de la transferencia nuclear a citoplasmas de ovocitos de animales prepúberes. En esta experiencia se transfirieron los núcleos de ovocitos de animales adultos a citoplasmas provenientes de ovocitos de animales prepúberes y viceversa, demostrando que el bajo potencial de desarrollo de los ovocitos estaba directamente relacionado con el citoplasma de los ovocitos de animales prepúberes.

Damiani *et al*. (1996) atribuyeron la baja capacidad de desarrollo de los ovocitos de ternera a una maduración citoplasmática incompleta o retrasada. Estos autores evidenciaron que se produce un retraso en la migración y reorganización de los organelos celulares tras la maduración, así como un incremento de las configuraciones anormales de cromosomas y microtúbulos tras la fecundación *in vitro*, debidos principalmente a errores en la formación del aster espermático y a una asincronía en la formación de los pronúcleos. Estas modificaciones podrían ser compatibles con la fecundación pero comprometerían la viabilidad embrionaria evitando que estos puedan desarrollarse normalmente. Por otra parte Armstrong (2001) observó que los ovocitos de

animales prepúberes presentan una zona pelúcida más delgada, vesículas intracitoplasmáticas de pequeño tamaño y un menor número y tamaño de clusters de gránulos corticales.

Otro factor que influye en la capacidad de los ovocitos de animales prepúberes para llevar a cabo la maduración nuclear y citoplasmática es el tamaño del folículo del que provienen. Así, los ovocitos bovinos procedentes de folículos de diámetro superior a 6 mm son capaces de madurar adecuadamente y desarrollarse hasta blastocistos, mientras que aquellos ovocitos procedentes de folículos con un diámetro entre 2 y 6 mm, presentan un menor porcentaje de formación de blastocistos (Lonergan *et al.*, 1994). Por otra parte, los ovocitos procedentes de folículos de menos de 2 mm de diámetro son incapaces de desarrollarse más allá del estadio de 8 células (Pavlok *et al.*, 1992). Con el incremento del tamaño del ovocito, incrementa su capacidad para desarrollarse hasta blastocisto, observándose que el tamaño óptimo de un ovocito inmaduro con capacidad de desarrollo es de 135 µm (Fair *et al.*, 1995; Arlotto *et al.*, 1996; Harada *et al.*, 1997). La importancia de este hecho radica en que el diámetro medio de los ovocitos de ternera (118 ± 1 µm) es ligeramente inferior al del ovocito de una vaca adulta (123 ± 1 µm), lo que a su vez se ve reflejado en el menor tamaño de los folículos de los cuales provienen (Armstrong, 2001). En estudios realizados por Fair et al. (1995), se observó que el tamaño de los ovocitos de ternera tienen una correlación positiva con el tamaño folicular. De esta forma, el potencial de desarrollo (incluyendo la capacidad para completar la maduración meiótica y el desarrollo embrionario después de la fecundación) aumenta con el incremento del tamaño del ovocito.

A nivel proteico, Lèvesque y Sirard (1994) evidenciaron la existencia de diferentes perfiles proteicos entre ovocitos bovinos procedentes de animales prepúberes y adultos, observándose que los ovocitos de terneras prepúberes presentan perfiles similares a los obtenidos en ovocitos de folículos atrésicos. Estos datos fueron confirmados por Khatir *et al.* (1996) que demostraron la existencia de pequeñas diferencias en los patrones de síntesis de nuevas proteínas en ovocitos de terneras prepúberes. Salamone *et al*. (2001) observaron que la actividad del MPF y la MAPK era menor en ovocitos de ternera que en ovocitos de vacas, evidenciando que la síntesis y almacenaje de reguladores de la maduración esta comprometido en los ovocitos de ternera. Por estas observaciones, Salamone *et al*. (2001) hipotetizaron que la falta de capacidad para llevar a cabo el

desarrollo embrionario por parte de los ovocitos prepúberes se debía, en gran medida, al fallo o incapacidad de estos ovocitos para completar la maduración citoplasmática.

A nivel metabólico, se han observado diferencias significativas en el metabolismo glucosídico y oxidativo entre ovocitos de animales prepúberes y adultos. En esta línea, Gandolfi *et al.* (1998) observaron que los ovocitos de ternera presentaban un menor metabolismo oxidativo del piruvato y la glutamina durante las primeras 3 horas de cultivo *in vitro*. Así mismo, Steeves *et al.* (1999) observaron que los ovocitos de animales prepúberes presentaban un menor metabolismo glucosídico durante las primeras 12 horas de maduración *in vitro*.

Las evidencias descritas anteriormente sugieren que los ovocitos de terneras son más sensibles y, por tanto, menos tolerantes a condiciones de cultivo subóptimas. Por ello, para que la producción de embriones *in vitro* procedentes de animales prepúberes sea más eficiente se requiere la optimización de las condiciones de cultivo, además de identificar las deficiencias intrínsecas que propician su fallo en el desarrollo.

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CAPÍTULO III:

EFFECTS OF VITRIFICATION IN OPEN PULLED STRAWS ON THE CYTOLOGY OF *IN VITRO* MATURED PREPUBERTAL AND ADULT BOVINE OOCYTES.

3.1. Abstract.

This study was designed to evaluate the effects of the cryopreservation of oocytes obtained from prepubertal calves or adult cows on chromosome organization, spindle morphology, cytoskeleton structures, and the ability of fertilized oocytes to develop to the blastocyst stage. Once *in vitro* matured (IVM), the oocytes were divided into 3 groups according to whether they were: 1) left untreated (control); 2) exposed to cryoprotectant agents (CPAs); or 3) cryopreserved by the open-pulled-straw (OPS) vitrification method. After thawing, oocyte samples were fixed, stained using specific fluorescent probes and examined under a confocal microscope. The remaining oocytes were fertilized, and cleavage and blastocyst rates recorded. After vitrification or CPA exposure, significantly higher proportions of oocytes showed changes in spindle morphology compared to the control group. The spindle structure of the adult cow IVM oocytes was significantly more resistant to the OPS vitrification process. Vitrification of oocytes from calves or adult cows led to significantly increased proportions of oocytes showing discontinuous or null actin staining of the cytoskeleton compared to non-treated controls. Oocytes only exposed to the cryoprotectants showed a similar appearance to controls. A normal distribution of actin microfilaments was observed in both calf and adult cow oocytes, irrespective of the treatment. Cleavage and blastocyst rates were significantly lower for vitrified versus non-treated oocytes. Oocytes obtained from adult cows were more sensitive to CPA exposure, while the vitrification procedure seemed to have more detrimental effects on the calf oocytes.

3.2. Introduction.

Ovaries obtained from the slaughterhouse have become a widely used source of oocytes for procedures such as IVF, cloning or other reproductive technologies. Given the limited time an oocyte remains viable and the limited number of oocytes that can be collected on any given day, the successful cryopreservation of mammalian oocytes is of great interest for basic research and commercial applications. Thus far, the oocytes of some mammalian species have been cryopreserved successfully through slow freezing procedures or vitrification, but rates of subsequent fertilization and development are much lower than those obtained using fresh oocytes. The first successful blastocysts and offspring achieved from cryopreserved oocytes in cattle were obtained by slow-rate

freezing (Lim *et al.*, 1991). This was followed by reports of similar developmental rates and offspring after vitrification (Hamano *et al.*, 1992). Since these initial attempts, new vitrification techniques for the cryopreservation of bovine oocytes have emerged (Martino *et al.*, 1996; Arav and Zeron, 1997; Vajta *et al.*, 1998). The open-pulled-straw (OPS) method developed by Vajta et al. uses a minimum amount of vitrification solution and is reported to achieve blastocyst rates of 25% for *in vitro* matured/fertilized bovine oocytes. Moreover, live offspring were produced after the transfer of embryos OPS-vitrified a second time at the blastocyst stage (Vajta *et al.*, 1998).

The loss of developmental potential after cryopreservation makes mammalian oocytes probably one of the most difficult cell types to cryopreserve. Indeed, the survival and developmental capacity of the cryopreserved oocyte are greatly impaired, probably as a consequence of morphological and cytological damage induced by the cryopreservation process. Ultrastructural studies on vitrified bovine oocytes have revealed that intercellular communication between the cumulus cells and oocyte might be interrupted and that the zona pellucida may be modified by premature cortical granule release (Fuku *et al.*, 1995). Changes in the structure of the cytoskeleton, mitochondria, cortical granules and nucleoli have also been observed in bovine oocytes (Saunders and Parks, 1999; Wu *et al.*, 1999; Hyttel *et al.*, 2000). However, the factors most likely to affect the success of bovine oocyte cryopreservation are the particular structural and functional characteristics of the oocyte, such as its size (Liebermann *et al.*, 2002), cumulus-oocyte complex, maturation status (Hochi *et al.*, 1998; Le Gal and Massip, 1999; Men *et al.*, 2002) and the dynamics of subcellular organelles during meiosis (reviewed in Shamsuddin *et al.*, 1996).

It is generally accepted that prepubertal oocytes are less developmentally competent than oocytes retrieved from adult cows (reviewed in Gandolfi *et al.*, 2000). Although prepubertal calf oocytes give rise to similar rates of fertilization and cleavage to those achieved using adult cow oocytes, their capacity to develop to the blastocyst stage is relatively lower (Revel *et al.*, 1995; Salamone *et al.*, 2001) and it would seem that embryos from calf oocytes are less capable of establishing pregnancies (Khatir *et al.*, 1998). According to Salamone et al. (Salamone *et al.*, 2001) this reduced developmental competence is attributable to the failure or inability of calf oocytes to complete ooplasmic maturation.

The aim of our study was to evaluate the effects of vitrification by the OPS method or exposure to cryoprotectant agents (ethylene glycol, EG and dimethylsulfoxide, DMSO) on *in vitro* matured oocytes obtained from calves and adult cows. Results were evaluated in terms of effects on chromosome, microtubule and microfilament distributions and the ability of oocytes to undergo fertilization and development to the blastocyst stage.

3.3. Materials and Methods.

In vitro embryo production.

The method used for the *in vitro* maturation and fertilization of the oocytes has been described elsewhere (Rizos *et al.*, 2001). Briefly, ovaries from slaughtered prepubertal and adult cows were transported from a local abattoir to the laboratory in PBS at 35º-37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2- to 6-mm follicles. After three washes in modified PBS (PBS supplemented with 36µg/mL pyruvate, 50 µg/mL gentamycin and 0.5 mg/mL BSA), groups of up to 50 COCs were placed in a 500-µL maturation medium (TCM-199 supplemented with 10% (v:v), fetal calf serum (FCS), 10 ng/mL EGF and 50 µg/mL gentamycin) in four-well plates and cultured for 24 h at 38.5ºC in a 5% CO_2 humidified air atmosphere.

For *in vitro* fertilization, COCs were washed four times in PBS and then in the fertilization medium before being transferred in groups of up to 50 into four-well plates containing 250 µL of fertilization medium per well (Tyrode medium supplemented with 25mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 µg/mL heparin-sodium salt). Motile spermatozoa were obtained by centrifuging frozen-thawed sperm from Asturian bulls on a discontinuous Percoll density gradient (2.5 mL 45% Percoll over 2.5 mL 90% Percoll) for 8 min at 700 x g at room temperature. Viable spermatozoa, collected at the bottom of the 90% fraction, were washed in Hepes-buffered Tyrode and pelleted by centrifugation at 100 x g for 5 min. Spermatozoa were counted in a hemocytometer and diluted in the appropriate volume of fertilization medium to give a final concentration of 2 x 10^6 spermatozoa/ml. A 250-µL aliquot of this suspension was added to each fertilization well to obtain a final

concentration of 1 x 10^6 spermatozoa/mL. Plates were incubated for 24 h at 38.5ºC in a 5% CO_2 humidified air atmosphere. Semen from the same bulls was used in all the experiments.

Embryo culture was performed in microdroplets on a seeded layer of granulosa cells in TCM-199 supplemented with 10% (v:v) FCS. At approximately 22 h post-insemination (hpi), presumptive zygotes were denuded by gentle vortexing and washed four times in PBS and twice in the culture medium before being transferred to a granulosa cell monolayer. Co-culture was conducted for 8 days at 38.5ºC in a 5% CO_2 humidified air atmosphere. Every 48 h during culture, approximately half of the culture medium was replaced with fresh medium. Cleavage rates were recorded at 48 hpi and the number of blastocysts was determined on post-insemination Days 7, 8 and 9.

Oocyte vitrification.

The vitrification procedure was essentially as described by Vajta et al. (Vajta *et al.*, 1998). All manipulations were performed on a 41ºC hot plate in a room at 25º - 27ºC. Twenty two hours after the onset of maturation, oocytes were denuded by gently pipetting in PBS until only two to four layers of cumulus cells remained on the surface. Oocytes were first equilibrated in a holding medium (TCM199-HEPES supplemented with 20% FCS) for 5 min and initially dehydrated by 30 sec exposure to 1mL 10% EG + 10% DMSO in TCM199-HEPES + 20% FCS. The oocytes were then transferred to 20-µL droplets of 20% EG + 20% DMSO in TCM199-HEPES + 20% FCS + 0.5M sucrose. Straws were loaded with three to five oocytes each by touching the surface of a 1-1.5-µL droplet of vitrification solution containing the oocytes with the narrow tip of the OPS. The loaded straw was then directly plunged into liquid nitrogen within 25 sec. Warming was performed by exposing the OPS straw to air for 3 sec prior to directly immersing the narrow tip of the straw into 1.2 mL of TCM199-HEPES medium containing 20% FCS + 0.25M sucrose. The oocytes were directly expelled into the medium after the vitrified medium became liquid. After about 5 min, the oocytes were transferred into 0.15 M sucrose for further rehydration. They were then washed in the 0.8 mL of holding medium for 5 min and *in vitro* maturation continued in the original dish for a further 2 hours. A sample of the vitrified oocytes was fixed and stained using

specific fluorescent probes before observation under a laser-scanning confocal microscope. The remaining oocytes were fertilized, and cleavage and blastocyst rates recorded.

Oocyte immunostaining.

Samples of oocytes from the experimental groups (see Experimental design) were fixed in 2% formaldehyde PBS, permeabilized using Triton X-100 (0.2% in PBS) and simultaneously immunostained for actin filaments, tubulin and chromatin detection (Boiso *et al.*, 2002). Fixation and subsequent incubations were performed at 37ºC.

For immunostaining, fixed oocytes were incubated with the anti-α-tubulin monoclonal antibody (1:250) for 1.5 h, followed by incubation with anti-mouse IgG antibody-biotin (1:5000) for 1 h, avidin-Cy5 for 30 min and falloidin-fluoroscein isothiocyanate (FICT) conjugate (1:1000) for 1 h. Chromosomes were counterstained by incubating the oocytes in propidium iodide (5mg/mL) for 30 min. Between incubations, the oocytes were washed three times in pre-warmed PBS for 5 min.

Groups of five oocytes were mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and then covered with an antifade mounting medium (glycerol-n-propyl-gallate and sodium azide in PBS). The preparation was sealed with nail varnish and stored refrigerated and protected from light until observation within the following two days. The actin filaments, tubulin and chromatin, stained with FICT, Cy5 and propidium iodide, respectively, were examined under a laser-scanning confocal microscope (Leica TCS-SP2 - AOBS) provided with an argon-krypton laser. Images were recorded on a host computer.

Spindle morphology was regarded as normal when the structure was symmetric, barrel-shaped, lacked astral microtubules and the diameter of the metaphase plate was longer than the pole-pole distance. Chromosome organization was regarded as normal when the chromosomes were arranged on a compact metaphase plate at the equator of the structure. Microtubules could also be seen in the first polar body of the oocyte. Spindle structure was regarded as abnormal when there was microtubule disruption, partial or total disorganization, or a complete lack of microtubules. Chromosome organization

was regarded as abnormal when chromosomes were dispersed or had an aberrant, less condensed appearance. Details of the abnormal patterns found are provided in Figure 1.

The cytoskeleton actin band was considered normal when an evenly stained layer of actin was observed immediately beneath the plasma membrane. Changes in the appearance of the cortical actin band were classified as abnormal when diffuse or discontinuous actin staining was observed, and the band was considered as missing when no staining was seen (Fig.1).

Experimental design.

Oocytes (from adult cows/calves) were randomly assigned to one of three experimental groups: (1) control oocytes matured *in vitro* for 24 h (N = 133/106); (2) oocytes matured for 22 h and exposed to CPAs (N = 138/106); (3) oocytes matured for 22 h and vitrified by the OPS method in the presence of CPAs (N = 150/86). Oocytes in groups 2 and 3 were left to mature for an additional 2 hours after treatment. The experiment was performed as three replicates.

Statistical analysis.

The χ^2-test was used to compare data among the ex

perimental groups. The level of statistical significance was set at $P < 0.05$.

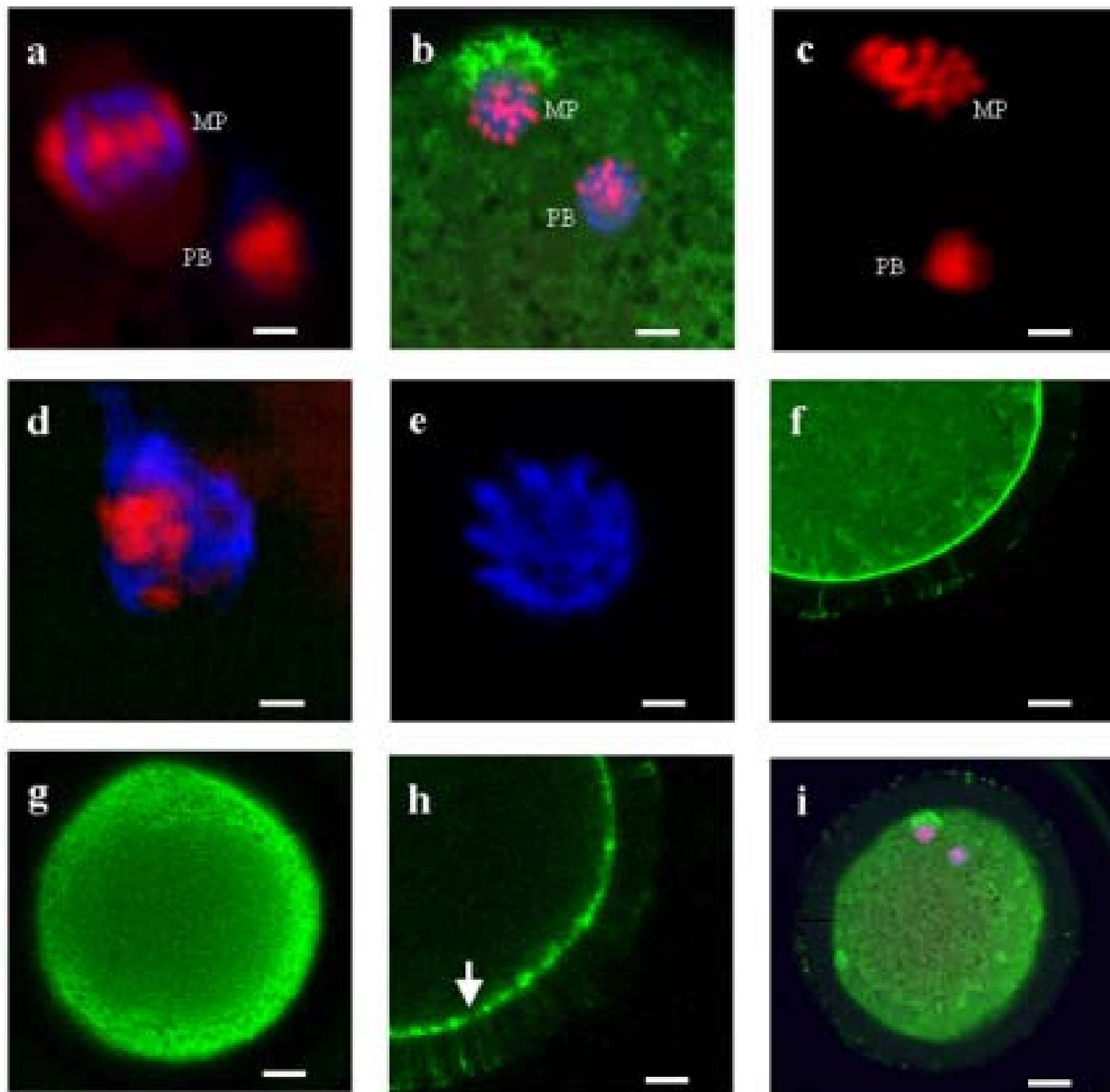

Figure 1. Confocal laser-scanning photomicrographs of bovine oocytes after CPA exposure or vitrification. Oocytes were immunocytochemically stained using an anti-α-tubulin monoclonal antibody and avidin-Cy5 to visualize the microtubules (blue), and counterstained with propidium iodide to visualize chromosomes (red) and phalloidine-fluoroscein isothiocyanate to visualize actin filaments (green). (a) Normal barrel-shaped Metaphase II spindle with compact chromosomes arranged at the equator of the structure; the first polar body can also be observed (scale bar=3.3 μm). (b) Abnormal spindle structures associated with disorganized chromosomes (scale bar=6.1 μm). (c) Dispersed chromosomes with no obvious spindle (scale bar=3.3 μm). (d) Abnormal spindle structures associated with disorganized, less condensed chromosomes (scale bar=2.6 μm). (e) Abnormal microtubule configuration (scale bar=2.6 μm). (f-h) Photomicrographs of oocytes for which only the actin (green) emission channel was active. (f) Normal distribution of microfilaments: an evenly stained layer of actin was observed immediately beneath the plasma membrane (scale bar=10.9 μm). (g) Oocyte showing a diffuse actin band (scale bar=15.4 μm). (h) Discontinuous distribution of microfilaments: white arrow points to an area showing no actin signal (scale bar=12 μm). (i) Oocyte with no actin band staining (scale bar=18.1 μm). MP: Metaphase plate. PB: Polar body

3.4. Results.

Cleavage and blastocyst production rates

The calves used were of a mean age less than 9 months, whereas adult cows were over 24 months. No significant differences were observed in cleavage rates between oocytes from calves and cows in the control groups (Table 1) although the blastocyst rate of calf oocytes was significantly lower (P<0.05) than for the cow oocytes (7.8% vs 33%).

Regardless of the type of oocyte, cleavage and blastocyst rates were significantly lower (P<0.05) for oocytes exposed to CPAs or vitrified in OPS, compared to controls. When the cleavage and blastocyst rates of calf and cow oocytes subjected to vitrification were compared, a significantly lower proportion of the calf oocytes underwent cleavage (13.5% vs 27.5%) and, while 2.5% of the adult cow oocytes developed into blastocysts, none of the calf oocytes reached the blastocyst stage.

Table 1. Effects of CPA exposure and vitrification by the OPS method on cleavage and embryo development

	Cow oocytes (%)			Calf oocytes (%)		
	n	Cleavage	Blastocyst	*n*	Cleavage	Blastocyst
Control	100	67 (67) a	33 (33.0) a,d	77	48 (62.3) a	6 (7.8) a,e
CPA control	99	47 (47.5) b	5 (5.1) b	76	39 (51.3) a	2 (2.6) a
OPS	120	33 (27.5) c,d	3 (2.5) b,d	52	7 (13.5) b,e	0 (0) b,e

a, b, c: Different letters within a column indicate statistically significant differences (P<0.05); d,e : Different letters within a row indicate statistically significant differences (P<0.05).

Microtubule and chromosome changes in vitrified oocytes

Table 2 shows the details observed in spindle and chromosome organization. Normal spindle and chromosome configurations were observed in 51.3% of the CPA-exposed and 40% of the vitrified cow oocytes, differing significantly from the 87.8% recorded for control oocytes. Calf oocytes exposed to CPA showed no significant differences compared to controls (63.3% vs 82.8%, respectively), while a significantly reduced percentage of those undergoing vitrification showed a normal spindle configuration (35.3%). Both exposure to CPA and vitrification led to increased percentages of oocytes with abnormal spindles compared to those lacking spindles.

Table 2. Effects of CPA exposure and OPS vitrification of IVM cow and calf oocytes on spindle morphology (chromosome arrangement and microtubule distribution)[a]

	Cow oocytes (%)				Calf oocytes (%)			
	n	Normal	Abnormal	Missing	*n*	Normal	Abnormal	Missing
Control	33	29 (87.8) a	2 (6.1)	2 (6.1)	29	24 (82.8) a	4 (13.8)	1 (3.4)
CPA control	39	20 (51.3) b,c	16 (41.0)	3 (7.7)	30	19 (63.3) a,d	8 (26.7)	3 (10.0)
OPS	30	12 (40.0) b,c	17 (56.7)	1 (3.3)	34	12 (35.3) b,d	14 (41.2)	8 (23.5)

a, b: Different letters a column indicate statistically significant differences (P<0.05). c,d : Different letters within a row indicate statistically significant differences (P<0.05).

[a] Saunders and Parks, 1999

Changes in the actin filaments of vitrified oocytes

In most of the untreated oocytes (90.9% and 96.5% for oocytes from cows and calves, respectively), actin showed a compact and homogeneous staining pattern at the periphery of the oolema. CPA exposure or vitrification increased the percentages of calf and cow oocytes with an abnormal actin band. No differences in the proportions of oocytes showing a normal actin band were recorded between cow and calves oocytes in

the control group (90.0% versus 96.5%), whether exposed to CPA (79.4% versus 80%) or vitrified (70% versus 70.6%) (Table 3).

Table 3. Effects of CPA exposure and OPS vitrification of IVM calf and cow oocytes on microfilament distribution[a]

	Cow oocytes (%)				Calf oocytes (%)			
	n	Normal	Abnormal	Missing	*n*	Normal	Abnormal	Missing
Control	33	30 (90.9) a	0 (0)	3 (9.1)	29	28 (96.5) a	1 (3.5)	0 (0)
CPA control	39	31 (79.4) ab	4 (10.3)	4 (10.3)	30	24 (80.0) ab	4 (13.3)	2 (6.7)
OPS	30	21 (70.0) b	4 (13.3)	5 (16.7)	34	24 (70.6) b	4 (11.8)	6 (17.6)

[a, b]: Values with different letters are significantly different ($P<0.05$).

[a] Saunders and Parks, 1999

3.5. Discussion.

In the present study, we evaluated the effects of vitrifying oocytes recovered from calves and adult cows on chromosome, microtubule and microfilament organization and then determined the ability of these oocytes to be fertilized and develop to the blastocyst stage.

The rates of cleavage and blastocyst development achieved by the calf and cow oocytes following vitrification were significantly lower than the rates recorded for non-treated oocytes. Moreover, calf oocytes were found to be more sensitive to freezing injury than cow oocytes. Since the production of the first blastocyst-stage embryo following the IVF of frozen-thawed matured bovine oocytes (Lim *et al.*, 1991), many research teams have tried to improve cryopreservation procedures for bovine oocytes. Recent reports on novel vitrification methods argue that technological innovations might greatly improve biological survival following cryopreservation. The OPS method developed by Vajta et al. (1998), besides being simple and inexpensive, achieves a vastly increased cooling speed by reducing the volume to be vitrified and narrowing the insulating layer between

the cooling agent and the vitrification solution. Using this method, these authors have reported rates of up to 50% cleavage and up to 25% blastocyst development after vitrification. Our cleavage rates and blastocyst yields after the vitrification of cow and calf oocytes are lower than those obtained by Vajta et al. (1998), but similar to those reported by other authors (Men *et al.*, 2002). Moreover, in accordance with the results of Martino et al. (1996) and Rho et al. (2002), control oocytes exposed to CPA without further cooling showed reduced development over controls, suggesting that osmotic shock plays an important role in the success of cryopreservation procedures.

Calf oocytes are less competent to develop than those harvested from adult cows (reviewed in Gandolfi *et al.*, 2000). Although fertilization and cleavage rates do not differ greatly for calf and cow oocytes, blastocyst yields are significantly reduced for calf oocytes (Revel *et al.*, 1995; Khatir *et al.*, 1996). This deficient developmental capacity of calf oocytes is most likely due to the abnormal cytoplasmic maturation of these oocytes. Both our cow and calf oocytes were matured *in vitro*, such that the differences observed were not attributable to our IVM system but more likely the result of an intrinsic deficiency of calf oocytes. It is also possible, however, that calf oocytes, because of their immaturity at the time of collection, require a modified medium to achieve developmental competence. Damiani et al. (1996) reported that calf oocytes show a delay in organelle migration (mainly cortical granules) following *in vitro* maturation, as well as abnormal chromatin and microtubule configurations. Nevertheless, in the present study, similar percentages of untreated calf and cow oocytes showed a normal spindle configuration and distribution of actin microfilaments.

Exposure to the cryoprotectants EG and DMSO had a drastic effect on the arrangement of microtubules and chromosomes in cow oocytes, while the vitrification procedure seemed to more severely affect the spindle configuration of calf oocytes. Approximately 64% of the vitrified calf oocytes and 60% of the vitrified cow oocytes appeared to have abnormal or missing spindles compared to control oocytes, while in the manipulated controls, abnormal spindle patterns were detected in ~ 37% of the calf and ~ 48% of the cow oocytes. Several authors have described that the main hurdle in developing successful protocols for the cryopreservation of mammalian oocytes is being able to preserve the integrity of the meiotic spindle when the oocytes are cooled (Eroglu *et al.*, 1998). Temperature fluctuations directly affect the cytoskeletal and chromosome

organization of mature bovine (Aman and Parks, 1994; Saunders and Parks, 1999) and human oocytes (Almeida and Bolton, 1995). The main consequence of cooling is pronounced depolymerization and the disappearance of microtubule organizing centers (Webb *et al.*, 1986). Chilling leads to the disassembly of spindle fibers within minutes, followed by an equally rapid reassembly of the spindle after the return to normal temperatures (Inoue, 1981). Magistrini and Szöllösi (1980) reported that the meiotic spindles of mouse oocytes were sensitive to cooling, with complete disassembly occurring after 45-60 min at 0ºC. The effects of cooling on the spindle appeared to be reversible in the mouse oocyte, with normal spindle formation occurring after step-wise re-warming. Pickering et al. (Pickering *et al.*, 1990) found that the meiotic spindle of human oocytes completely disassembled, and this was accompanied by chromosomal dispersion in 60% of the oocytes after 30 min at room temperature. This effect appeared to be reversible in only 25%-50% of the oocytes. The meiotic spindle becomes completely disassembled when *in vitro*-matured bovine oocytes are maintained for 10-20 min at 4ºC (Richardson and Parks, 1992). When pig oocytes were kept for 5 min at 4ºC, microtubules in the spindles of most oocytes partially or completely disassembled (Liu *et al.*, 2003).

Cryoprotectants are known to induce changes in microtubule organization in several species, including the mouse (Vincent and Johnson, 1992; Cooper *et al.*, 1996), rabbit (Vincent *et al.*, 1989), human (Sathananthan *et al.*, 1987) and cow (Hyttel *et al.*, 2000). The exposure of bovine oocytes to EG did not affect spindle arrangement after 20 min re-warming, but a lower percentage of oocytes with abnormal spindle configuration was seen after 1 h and 3 h (Saunders and Parks, 1999). In our study, effects on the normal spindle pattern were similarly observed after 2 h re-warming in oocytes exposed to EG + DMSO. At room temperature, the addition of a cryoprotectant (propanediol or DMSO) to rabbit oocytes led to disorganization of spindle microtubules (Vincent *et al.*, 1989). In mouse oocytes, a similar effect of DMSO (Jonhson and Pickering, 1987; Van der Elst *et al.*, 1988) and propanediol (Van der Elst *et al.*, 1988) has been noted, and a consequent dispersal of chromosomes is often seen in both species.

In this study, exposure to cryoprotectants led to no substantial disruption of microfilament organization in either cow or calf oocytes. Williams et al. (Williams *et al.*, 1992) observed that microfilament organization in bovine oocytes loaded with EG

or PrOH was similar to that in untreated oocytes and Saunders and Parks (Saunders and Parks, 1999) described the normal structure of the actin band within 1 h to 3 h post-thawing of oocytes exposed to EG. These results confirm our observations of only a discrete effect of EG +DMSO on the distribution of actin. Vitrification was, however, found to modify the organization of actin filaments. Freezing has also been described to causes changes in the organization of cytoskeleton actin in rabbit and mouse oocytes (Vincent *et al.*, 1989; George and Johnson, 1993), although these changes were often reversible upon thawing. Dramatic effects on cytoskeletal actin were observed after freezing bovine oocytes (Saunders and Parks, 1999). Because of the association of microfilaments with other structures, it is possible that their disruption is the result of damage to another cell component such as the plasma membrane or mitochondria (Didion *et al.*, 1990; Fuku *et al.*, 1995). Disruption of the cytoskeleton may be intrinsic to the changes in shape and shrinkage related to cryopreservation procedures, which in turn may lead to irreversibly changes in the structure of the plasma membrane or cytoskeleton so that it can no longer adjust to changing conditions, including those associated with fertilization. Thus, even when the normal microfilament distribution of oocytes is restored after CPA exposure and vitrification, irreversible alterations to other cell components may already have occurred such as the early release of cortical granule enzymes and zona hardening. These changes may either prevent fertilization completely or incompletely block polyspermy, both leading to decreased cleavage rates after insemination.

In conclusion, the vitrification of bovine oocytes at the MII stage by the OPS method produces biological changes in the oocytes after thawing, reflected by subsequently impaired fertilization and embryo development. Oocytes retrieved from adult cows were found to be more sensitive to exposure to CPAs, while vitrification seemed to have worse effects on calf oocytes. In spite of these alterations, however, a certain proportion of the vitrified oocytes were capable of developing into embryos.

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CAPÍTULO IV:

EFFECTS OF ROSCOVITINE ON THE NUCLEAR AND CYTOSKELETAL COMPONENTS OF CALF OOCYTES AND THEIR SUBSEQUENT DEVELOPMENT

4.1. Abstract .

Roscovitine, a potent inhibitor of M-phase promoting factor kinase activity, was used to maintain calf oocytes at the germinal vesicle stage for a 24-h culture period. Cumulus-oocyte complexes were aspirated from slaughterhouse calf ovaries and cultured for 24 h in TCM199 containing different levels of roscovitine (12.5, 25, 50 and 100 μM). After this culture period, the oocytes were either fixed immediately or cultured for a further 24 h in conditions permissive of maturation. After fixing a sample of these oocytes, the remaining oocytes were subsequently fertilized and cleavage and blastocyst rates were recorded. Oocytes cultured in the presence of roscovitine, at all the concentrations tested, were significantly blocked at the germinal vesicle stage. The inhibitory effect varied according to the dose, with 50 μM and 100 μM roscovitine being the most efficient concentrations, producing developmental arrest at the GV stage in over 60.0% of oocytes. However, this inhibitory effect of roscovitine was fully reversible since over 73% of the oocytes cultured for 24 h in the presence of 50 μM roscovitine reached the metaphase II stage after a further 24 h of culture in a permissive medium. Cleavage rates and blastocyst yields were not significantly different for oocytes cultured under 50 μM roscovitine inhibition compared to oocytes not subjected to prematuration culture (rates of 76.7% cleavage and 8.7% blastocysts for control oocytes compared to 69.8% and 6.3% respectively for oocytes pretreated with 50 μM roscovitine). The morphology of the meiotic spindle was typical of metaphase II in 75.8% and 82.1% of the oocytes reaching the metaphase II stage after pretreatment with 50 μM roscovitine compared to control, respectively. A normal distribution of actin filaments was observed in 97.0% and 98.2% of oocytes exposed to 50 μM roscovitine compared to control, respectively. These results demonstrate the feasibility of maintaining calf oocytes in artificial meiotic arrest without compromising their subsequent developmental competence.

4.2. Introduction.

The *in vitro* maintenance of oocytes at the germinal vesicle (GV) stage helps them acquire developmental competence. The capacity to preserve oocytes at this stage without producing damage serves to synchronize the resumption of meiosis such that all collected oocytes can complete the cytoplasmic maturation process, maximizing embryo production.

In vivo, meiotically competent oocytes are kept at the GV stage by follicular factors until the preovulatory gonadotropin surge. This gives them time to differentiate before ovulation. *In vitro*, all meiotically competent oocytes spontaneously re-enter the meiotic process as soon as they are removed from their follicles. Nevertheless, nuclear meiotic maturation of the oocytes in itself is insufficient to ensure subsequent embryo development. Thus, we hypothesized that if oocytes are cultured before maturation *in vitro* under conditions that arrest meiosis at the GV stage, this might offer them the opportunity to acquire greater developmental competence.

Calf oocytes have been reported to lack developmental competence compared to adult cow oocytes (Damiani *et al.*, 1996; Duby *et al.*, 1996; Khatir *et al.*, 1996; Presicce *et al.*, 1997). Although fertilization and cleavage rates do not differ greatly between calf and cow oocytes, blastocyst yields using calf oocytes are significantly reduced (Khatir *et al.*, 1996). This reduced developmental competence may be due to a failure or inability of calf oocytes to undergo both nuclear and cytoplasmic maturation.

Attempts have been made to establish for pharmacological (cycloheximide, 6-dimethylaminopurine, butyrolactone I, roscovitine) or physiological (theca cells) inhibitors aimed at achieving meiotic arrest (Richard *et al.*, 1997; Saeki *et al.*, 1997; Avery *et al.*, 1998; Kubelka *et al.*, 2000; Mermillod *et al.*, 2000). Roscovitine (ROS), a specific cdc2 kinase inhibitor, has been shown capable of reversibly inhibiting meiotic resumption in cow oocytes for 24 h with no negative effects on subsequent development to the blastocyst stage (Mermillod *et al.*, 2000). Similar results have been reported for pig oocytes (Krischek and Meinecke, 2001; Marchal *et al.*, 2001). Donnay et al. (2004), on the other hand, observed that a prematuration treatment with ROS prevented the progression of meiosis in calf oocytes but that this treatment led to a dramatic decrease in embryo development after fertilization.

Lonergan et al. (2003) noted ultrastructural changes in bovine oocytes maintained in meiotic arrest *in vitro* using ROS. These authors observed that incubation with ROS leads to swelling of mitochondrial cristae, degeneration of cortical granules and convolution of the nuclear membrane. Chromatin and cytoskeleton alterations in pig oocytes treated with ROS were assessed by Ju et al. (2003), who reported that the

resumption of meiosis and effects on GV chromatin and the cytoskeleton were reversible once the drug was removed.

The present study was designed to evaluate: the effectiveness of ROS in maintaining calf oocytes at the GV stage for 24 h; subsequent nuclear maturation rates after removal of the inhibitor; and the resulting developmental potential of these oocytes kept at the GV stage for 24 h. We also examined the changes experienced by both nuclear and cytoskeletal components, including the chromosomes, microtubules and microfilaments, of oocytes treated with ROS during *in vitro* maturation.

4.3. Materials and methods .

Oocyte collection and inhibition of meiotic resumption.

The method used for the *in vitro* maturation and fertilization of the oocytes has been described elsewhere (Rizos *et al.*, 2001). Briefly, ovaries from slaughtered prepubertal calves were transported from a local abattoir to the laboratory in PBS at 35ºC-37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2- to 10-mm follicles. After three washes in modified PBS (PBS supplemented with 36 μg/mL pyruvate, 50 μg/mL gentamicin and 0.5 mg/mL BSA), groups of up to 50 COCs were placed in 500 μL of maturation medium containing different levels of ROS (12.5, 25, 50 and 100 μM) in four-well plates and cultured for 24 h at 38.5ºC in a 5% CO_2 humidified air atmosphere. The maturation medium was comprised of TCM-199 supplemented with 10% (v:v), fetal calf serum (FCS), 10 ng/mL EGF and 50 μg/mL gentamicin. ROS was prepared as a 5mM stock solution in dimethyl sulfoxide and stored in aliquots at – 20 ºC until use.

In vitro maturation

To allow the arrested oocytes to resume meiotic maturation, COCs were rinsed several times in modified PBS to avoid carry-over of ROS into the oocyte maturation medium and then cultured in maturation medium for a further 24 hours at 38.5ºC in a 5% CO_2 humidified air atmosphere.

In vitro fertilization

For *in vitro* fertilization, the COCs were washed four times in PBS and then in the fertilization medium before being transferred in groups of up to 50 to four-well plates containing 250 µL of fertilization medium per well (Tyrode's medium supplemented with 25mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 µg/mL heparin-sodium salt). Motile spermatozoa were obtained by centrifuging frozen-thawed sperm from Asturian bulls on a discontinuous Percoll density gradient (2.5 mL 45% Percoll over 2.5 mL 90% Percoll) for 8 min at 700 x g at room temperature. Viable spermatozoa, collected at the bottom of the 90% fraction, were washed in Hepes-buffered Tyrode's and pelleted by centrifugation at 100 x g for 5 min. Spermatozoa were counted in a hemocytometer and diluted in an appropriate volume of fertilization medium to give a final concentration of 2 x 10^6 spermatozoa/mL. A 250-µL aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1 x 10^6 spermatozoa/mL. Plates were incubated for 24 h at 38.5ºC in a 5% CO_2 humidified air atmosphere. Semen from the same bulls was used in all the experiments.

In vitro culture

Embryo culture was performed in microdroplets on a seeded layer of granulosa cells in TCM-199 supplemented with 10% (v:v) FCS. At approximately 20 h post-insemination (hpi), presumptive zygotes were denuded by gentle vortexing and washed four times in PBS and twice in the culture medium before being transferred to a granulosa cell monolayer. Co-culture was conducted for 8 days at 38.5ºC in a 5% CO_2 humidified air atmosphere. Every 48 h during culture, approximately half of the culture medium was replaced with fresh medium. Cleavage rates were recorded at 48 hpi and numbers of blastocysts were determined on post-insemination Days 7, 8 and 9.

Experiment 1

COCs were aspirated from slaughterhouse calf ovaries and cultured for 24 h in TCM199 containing different levels of ROS (12.5, 25, 50 and 100 µM). After 24 h of inhibition, a

third of the oocytes were denuded, fixed overnight, lacmoid stained and observed under a phase contrast microscope to determine their nuclear status. The remaining oocytes (two-thirds) that had been incubated in ROS-containing medium were washed and transferred to 500 μL of maturation medium for a further 24 h, after which another third of the original number of oocytes was fixed and stained as above. The remaining oocytes (final third) were fertilized and cleavage and blastocyst rates were recorded. Another batch of oocytes not exposed to ROS before IVM was also fixed and stained after 24 h of maturation (Control oocytes). The experiment was replicated four times.

Experiment 2

COCs were aspirated from slaughterhouse ovaries and cultured for 24 h in TCM199 containing different levels of ROS (12.5, 25, 50 and 100 μM). Half of the oocytes in each group were fixed at 24 h in fixative for immunocytochemical labeling of chromosomes, microtubules and microfilaments. The remaining oocytes that had been incubated in ROS-containing medium were washed and transferred to 500 μL of maturation medium for further 24 h and then fixed and examined with the aid of a confocal microscope to evaluate the reversibility of cytoskeletal and nuclear alterations following the removal of ROS. A batch of oocytes not exposed to ROS before IVM was also fixed and stained after 24 h of maturation (Control oocytes). The experiment was replicated four times.

Evaluation of nuclear maturation stage

Oocytes were freed from their surrounding cumulus cells by repeated pipetting and washed three times in PBS. To evaluate meiotic progression, the oocytes were fixed in ethanol:acetic acid (3:1) overnight. The oocytes were mounted on microscope slides with vaseline, covered with a glass coverslip and stained with lacmoid. The stage of nuclear maturation was assessed by phase contrast microscopy (magnification, x 40).

Oocytes were scored as being at the: *germinal vesicle (GV) stage*, when their chromatin, either filamentous or slightly condensed, was enclosed in a nuclear membrane; *germinal vesicle breakdown (GVBD) stage*, when there was no visible nuclear membrane and

chromatin was condensed; *metaphase I (MI) stage*, when the chromatin was distributed at the equator of a large spindle; *anaphase I stage*, when homologous chromosomes could be seen to move away from each other and there was a visible spindle; *telophase I stage*, when homologous chromosomes were apart; and *metaphase II (MII) stage*, when the chromatin was located at the spindle and abstriction of the first polar body was detected.

Oocyte immunostaining

Samples of oocytes from the experimental groups (see above) were fixed in 2% formaldehyde PBS, permeabilized using Triton X-100 (0.2% in PBS), and simultaneously immunostained for actin filaments, tubulin and chromatin detection (Boiso *et al.*, 2002). Fixation and subsequent incubations were performed at 37ºC.

For immunostaining, fixed oocytes were incubated with the anti-α-tubulin monoclonal antibody (1:250) for 1.5 h, followed by incubation with anti-mouse IgG antibody-biotin (1:5000) for 1 h, avidin-Cy5 for 30 min and falloidin-fluorescein isothiocyanate (FICT) conjugate (1:1000) for 1 h. Between incubations, the oocytes were washed three times in pre-warmed PBS for 5 min.

Groups of five oocytes were mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and then stained with DAPI (125 ng/mL). The preparation was sealed with nail varnish and stored refrigerated and protected from light until observation within the following two days. A laser-scanning confocal microscope (Leica TCS-SP2 - AOBS) was used to examine actin filaments (FICT; excitation 488 nm), tubulin (Cy5; excitation 633 nm) and chromatin (DAPI; excitation 405 nm). Images were recorded on a computer.

Statistical analysis

The χ^2-test was used to compare data among the experimental groups. The level of statistical significance was set at $P < 0.05$.

4.4. Results.

Experiment 1

In this experiment, the oocytes were cultured in maturation medium alone or in medium supplemented with increasing ROS concentrations (12.5, 25, 50 and 100 µM). At the end of the culture period, nuclear maturation states were established as described. These results (mean of 4 replicates) are shown in Table 1. Oocytes cultured in the presence of ROS were significantly blocked at the GV stage. This inhibitory effect varied according to dose; 50 µM and 100 µM roscovitine were the most efficient concentrations producing meiotic arrest (58.3% and 64.7% of oocytes at the GV stage for 50 and 100 µM, respectively). ROS concentrations of 12.5 µM (6.8%) and 25 µM (24.5%) showed a reduced effect.

Table 1: Nuclear maturation status of calf oocytes cultured for 24 h in IVM medium supplemented with increasing concentrations of ROS.

ROS µM	No. oocytes	GV *n* (%)	GVBD *n* (%)	MI *n* (%)	MII *n* (%)	Ana-telophase I *n* (%)	Degenerated *n* (%)
Control	153	2 (1.3) a	3 (2.0) a	18 (11.8) a	114 (74.5) a	0 (0) a	16 (10.5) ab
12.5	88	6 (6.8) b	6 (6.8) a	30 (34.1) b	32 (36.4) b	2 (2.3) b	12 (13.6) ab
25	94	23 (24.5) c	20 (21.3) b	28 (29.8) b	7 (7.4) c	1 (1.1) b	15 (16.0) a
50	139	81 (58.3) d	35 (25.2) b	12 (8.6) ac	1 (0.7) d	0 (0) a	10 (7.2) b
100	85	55 (64.7) d	22 (25.9) b	3 (3.5) c	0 (0)	0 (0) a	5 (5.9) b

Values with different letters within each column are significantly different, $P<0.05$.

Table 2 shows the results obtained when the oocytes were cultured for an additional 24-h period in inhibitor-free culture medium and then fixed and stained to assess the reversibility of the meiotic inhibition elicited by ROS. These data indicate that the inhibitory effect of ROS was reversible, since for each pretreatment ROS concentration,

a higher proportion of oocytes at the MII stage was observed after the second culture period compared to the first. Indeed, the proportions of MII oocytes recorded after removal of the inhibitor for the 25 µM (68.8%), 50 µM (74.8%) and 100 µM (63.9%) concentrations were not significantly different to those observed for the control oocytes (74.5%). However, when oocytes were prematured in the presence of 12.5 µM ROS, a significantly lower percentage of oocytes reached the MII stage (46.9%) after removal of the inhibitor.

Table 2: Nuclear maturation status of calf oocytes cultured for 24 h in IVM medium supplemented with increasing concentrations of ROS and then transferred to inhibitor-free medium for an additional 24 h.

ROS µM	No. oocytes	GV *n* (%)	GVBD *n* (%)	MI *n* (%)	MII *n* (%)	Degenerated *n* (%)
Control	153	2 (1.3) a	3 (2.0) a	18 (11.8) ab	114 (74.5) a	16 (10.5) a
12.5	98	0 (0) b	1 (1.0) a	18 (18.4) a	46 (46.9) b	33 (33.7) b
25	77	0 (0) b	0 (0) b	9 (11.7) ab	53 (68.8) a	15 (19.5) ac
50	127	0 (0) b	4 (3.1) a	9 (7.1) bc	95 (74.8) a	19 (15.0) a
100	72	0 (0) b	2 (2.8) b	3 (4.2) c	46 (63.9) a	21 (29.2) bc

Values with different letters within each column are significantly different, $P<0.05$.

Table 3 shows the embryo development rates of control and ROS-treated oocytes. When prematured oocytes were matured in inhibitor-free medium, fertilized and the subsequent embryos were cultured up to the blastocyst stage, cleavage rates did not significantly differ for oocytes cultured under 50 µM ROS inhibition compared to oocytes not subjected to prematuration culture (69.8% vs 76.7%, respectively for oocytes treated with 50 µM ROS and control oocytes). Concentrations of 12.5 µM, 25 µM and 100 µM ROS yielded significantly lower cleavage rates with respect to untreated controls.

Blastocyst yields on Day 9 post-insemination were similar for control and 50 µM ROS-pretreated oocytes (8.7% and 6.3%, respectively). However, significantly lower proportions of blastocysts were obtained after fertilization of oocytes prematured in the presence of 25 µM (2.2%) or 100 µM ROS (3.3%) when compared to control oocytes, although these results were not significantly different to percentages obtained after prematuration treatment with 50 µM ROS.

Table 3. Effects of inhibiting meiotic resumption in calf oocytes using different ROS concentrations on subsequent developmental competence.

ROS µM	No. oocytes	Cleaved *n* (%)	Blastocysts		
			Day 7 *n* (%)	Day 8 *n* (%)	Day 9 *n* (%)
Control	343	263 (76.7)a	18 (5.2) a	30 (8.7) a	30 (8.7) a
12.5	242	105 (43.4) b	0 (0) b	0 (0) b	0 (0) b
25	357	179 (50.1) b	5 (1.4) a	6 (1.7) c	8 (2.2) b
50	252	176 (69.8) a	10 (3.9) a	14 (5.5) ac	26 (6.3) ab
100	242	123 (50.8) b	6 (2.4) a	8 (3.3) ac	8 (3.3) b

Values with different letters within each column are significantly different, $P<0.05$.

Experiment 2

Figure 1 shows the normal nuclear and cytoskeletal morphology of calf oocytes at the stages GV, GVBD, MI and MII. Germinal vesicle oocytes showed a variety of different chromatin configurations, ranging from very diffuse to various degrees of condensation. However, the chromatin was always confined to a defined area (the GV) and persisted as a single mass with no visible organization as distinct chromosomes. Oocytes at the GV stage also showed a complex network of thread-like, discretely-labeled microtubules. Microtubule organizing centres were evident, mainly around the nuclear membrane. Indeed, no marked differences in microtubule organization were observed among oocytes with different chromatin configurations. Microfilaments appeared in slightly greater amounts just beneath the plasma membrane.

As the oocytes reenter the meiotic process, germinal vesicles break down (GVBD stage) and the chromatin condenses into short, thick, distinct chromosomes. At this point, densely stained radial-like microtubules could be observed in close association with the recently condensed and distinguishable chromosomes. Microfilaments clearly concentrated in the cortical region of the oocyte.

During the metaphase stage of the first meiotic division, these highly condensed chromosomes aligned themselves along the center of the spindle, radially elongated microtubules formed the meiotic spindle and microfilaments concentrated in the cortical region of the oocyte.

In oocytes at the metaphase stage of the second meiotic division, the spindle structure always appeared at the periphery, oriented perpendicularly to the plasma membrane. Oocytes that reached the MII stage were classified as follows:

A) The spindle morphology of MII oocytes was classified as three categories: (1) *Normal spindles*. These were barrel-shaped with chromosomes clustered as a discrete bundle at the metaphase plate and microtubules crosssing the length of the spindle from pole to pole or extending from the spindle poles to the chromosomes; microtubules within the polar body had no discernable organization and instead appeared as an amorphous mass intertwined with chromatin occupying the perivitelline space. (2) *Abnormal spindles*. Microtubules were not organized as typical spindles or some microtubules were disassembled. (3) *Absence of the spindle*. No microtubules could be observed around the chromosomes. Detailed images of these normal and abnormal patterns are provided in Figure 1.

B) Chromosomal organization was classified as: (1) *Dispersed chromosomes*. These were scattered in the cytoplasm or dispersed in a few zones of the cytoplasm. (2) *Decondensed chromosomes*. Chromosomes with an aberrant, less condensed appearance. (3) *Absence of chromosomes*. No chromosomes were observed (Figure 1).

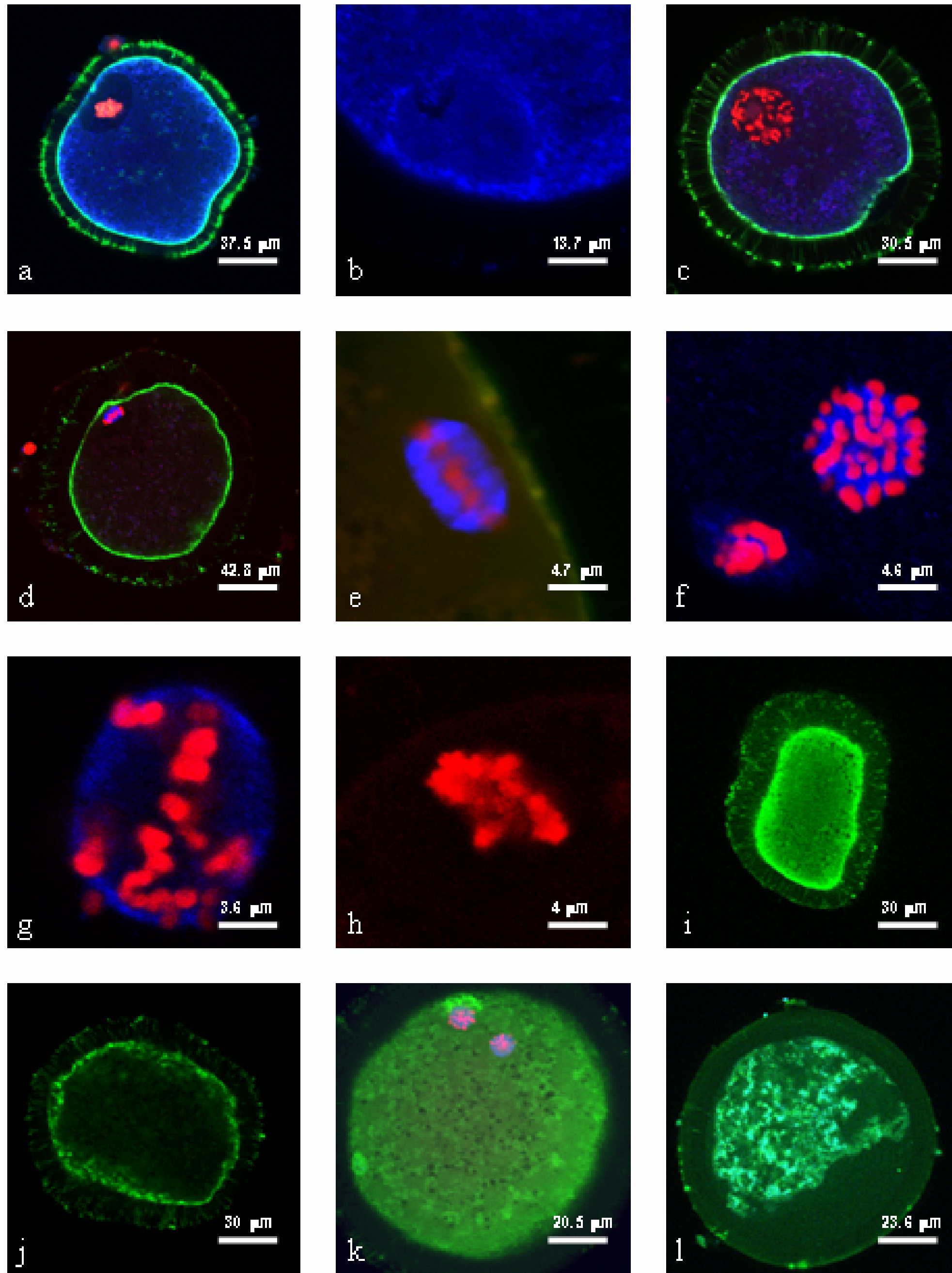

Figure 1. Confocal laser-scanning photomicrographs of bovine oocytes at different stages of maturation *in vitro* (a-e). Alterations observed in the spindle morphology, chromosome configuration and microfilament distribution of calf MII oocytes cultured in the presence of different ROS concentrations (f-l). Oocytes were immunocytochemically stained using an anti-α-tubulin monoclonal antibody and avidin-Cy5 to visualize the microtubules (blue), and counterstained with DAPI to visualize chromosomes (red) and phalloidine-fluoroscein isothiocyanate to visualize actin filaments (green). (a) Image of a germinal vesicle stage oocyte. Note the chromatin has yet to condense and microtubules form a network throughout the ooplasm. Microfilaments appear just beneath the plasma membrane of the oocyte. (b) Germinal vesicle stage oocyte showing microtubule organizing centers mainly distributed around the nuclear membrane. (c) A germinal vesicle oocyte at the breakdown stage. Note that microtubule asters can be seen in association with the now condensed chromatin strands, while the microfilaments are clearly concentrated inside the oocyte cortex. (d) Metaphase I stage oocyte with microtubules having now formed a clear meiotic spindle with chromosomes aligned at its equator. (e) Normal barrel-shaped metaphase II spindle with compact chromosomes arranged at the equator of the structure. Microtubules can be seen within the spindle. (f) Abnormal spindle structures associated with disorganized chromosomes. (g) Dispersed chromosomes and a spindle lacking microtubules between poles. (h) Chromosomes with an aberrant, less condensed appearance. Note the absence of microtubules. (i) Oocytes showing a diffuse actin band. (j) Discontinuous distribution of microfilaments. (k) Oocytes with no actin band staining. (l) Appearance of a degenerated oocyte. Note the scattered and disorganized aggregations of chromatin, microtubules and microfilaments clearly reflecting aberrant development or degeneration.

C) The cytoskeleton actin band in the MII oocytes was classified as four categories: 1) *Normal*. An evenly stained layer of actin appearing immediately beneath the plasma membrane. 2) *Diffuse*. Diffuse, irregular microfilament distribution, weakly stained. 3) *Discontinuous*. Interrupted staining of the actin band. 4) *Absence of actin band*. No microfilaments observed beneath the oolema (Figure 1).

When GV calf oocytes were incubated in medium containing ROS concentrations of 12.5-100 μM, blockage at the GV stage occurred in 64.6% and 63.2% of the oocytes at effective doses of 50 μM and 100 μM, respectively (Table 4). Table 5 shows the details observed in the spindle, the chromosome organization and the actin distribution of calf oocytes that reached the MII stage after 24 h of culture in the presence of different levels of ROS. When the distribution of microtubules and chromosomes was evaluated, 82.1% of the control oocytes and 76.9% of oocytes treated with 12.5 μM ROS showed normal spindles, 14.3% and 23.1% respectively had abnormal spindles and 3.6% and 0%, respectively, lacked spindles. In our evaluation of microfilament distribution, 98.5% of control oocytes and 100% of those treated with 12.5 μM ROS showed a normal actin band.

Table 4: Nuclear maturation status of calf oocytes cultured for 24 h in IVM medium supplemented with increasing concentrations of ROS.

ROS μM	No. oocytes	GV *n* (%)	GVBD *n* (%)	MI *n* (%)	MII *n* (%)	Degenerated *n* (%)
Control	72	0 (0) a	0 (0) a	12 (16.7) a	56 (77.8) a	4 (5.5) ab
12.5	34	1 (2.9) b	3 (8.8) b	13 (38.2) b	13 (38.2) b	4 (11.8) bc
25	32	6 (18.8) c	19 (59.4) c	6 (18.8) a	0 (0) c	1 (3.1) a
50	48	31 (64.6) d	4 (8.3) b	3 (6.3) c	0 (0) c	10 (20.8) cd
100	38	24 (63.2) d	7 (18.4) b	2 (5.3) c	0 (0) c	5 (13.1) bd

Values with different letters within each column are significantly different, $P<0.05$.

Table 5. Spindle morphology, chromosome configuration and actin distribution of MII stage calf oocytes after 24 h of culture with different ROS concentrations.

ROS μM	*n**	Spindle configuration, *n* (%)			Chromosome organization, *n* (%)			Actin distribution, *n* (%)			
		Normal	Abnormal	Absent	Dispersed	Decondensed	Absent	Normal	Diffuse	Discontinuous	Absent
Control	56	46 (82.1) a	8 (14.3) a	2 (3.6) a	7 (12.5) a	1 (1.8) a	2 (3.6) a	55 (98.2) a	1 (1.8) a	0 (0)	0 (0)
12.5	13	10 (76.9) a	3 (23.1) a	0 (0) b	2 (15.4) a	1 (7.7) a	0 (0) b	13 (100) a	0 (0) b	0 (0)	0 (0)
25	0	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0)	0 (0)
50	0	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0)	0 (0)
100	0	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0) b	0 (0)	0 (0)

*: Number of oocytes reaching the MII stage after 24 h of culture with different concentrations of ROS

Values with different letters within each column are significantly different, $P<0.05$.

Table 6 shows the nuclear progression of calf oocytes cultured for 24 h in IVM medium supplemented with increasing ROS concentrations and then in inhibitor-free medium for an additional 24 h. Prematuration treatment with 25 µM ROS gave rise to a significantly higher percentage (89.3%) of oocytes at the MII stage with respect to untreated oocytes (77.8%) and those pretreated with 12.5 µM (76.5%) and 50 µM (73.3%) ROS. Results after prematuration treatment with 100 µM ROS (78.6%) failed to differ significantly to those obtained for the remaining treatments.

Table 6: Nuclear maturation status of calf oocytes cultured for 24 h in IVM medium supplemented with increasing concentrations of ROS and then transferred to inhibitor-free medium for an additional 24 h.

ROS µM	No. oocytes	GV *n* (%)	GVBD *n* (%)	MI *n* (%)	MII *n* (%)	Degenerated *n* (%)
Control	72	0	0	12 (16.7) ab	56 (77.8) a	4 (5.5) a
12.5	51	0	0	10 (19.6) a	39 (76.5) a	2 (3.9) a
25	56	0	0	5 (8.9) b	50 (89.3) b	1 (1.8) a
50	45	0	0	6 (13.3) ab	33 (73.3) a	6 (13.3) b
100	56	0	0	8 (14.3) ab	44 (78.6) ab	4 (7.1) ab

Values with different letters within each column are significantly different, P<0.05.

Table 7 summarizes the results obtained when we evaluated the spindle morphology and chromosome organization of oocytes reaching the MII stage after removal of the inhibitor during *in vitro* maturation. Meiotic spindles and cytoplasmic microtubules showed the typical MII morphology in 75.8% of oocytes prematured with 50 µM ROS, not differing significantly from the control treatment (82.1%). The percentages of oocytes showing a normal spindle distribution decreased significantly for oocytes prematured in the presence of 25 µM (42.0%) and 100 µM ROS (52.3%). Concentrations of 12.5, 25 and 100 µM ROS led to significantly increased proportions of oocytes with an abnormal microtubule organization (38.5%, 34.0%, and 31.8%, respectively). Chromosome disorganization was observed in a higher proportion of

oocytes pretreated with all the ROS concentrations tested than in untreated controls (33.3%, 42.0%, 24.2% and 43.2% vs 12.5% for 12.5, 25, 50 and 100 μM ROS vs control, respectively). When oocytes were prematured with 25 μM ROS, a significantly higher percentage of chromosomal decondensation was observed when compared to the other treatments.

Our analysis of the cytoskeleton actin band in oocytes that reached the MII stage revealed a normal distribution of microfilaments in 98.2% of control oocytes and 97.0% of oocytes treated with 50 μM ROS. Concentrations of 12.5, 25 and 100 μM ROS produced a significant decrease in the proportions of oocytes with a normal microfilament distribution while these ROS levels led to a significant increase in the number of oocytes showing diffuse actin labeling (38.5%, 18.0% and 25.0% for 12.5, 25 and 100 μM ROS, respectively).

Degenerated Oocytes

After 24 h of ROS treatment, some of the oocytes were classified as degenerated. The proportion of degenerated oocytes ranged from 3.1% to 20.8%, depending on the treatment and was lowest (1.8% to 13.3%) after IVM in the inhibitor-free medium. Oocytes classified as degenerated showed aberrant chromatin and cytoskeletal patterns. In these degenerated oocytes, DNA was either not visible or visible only as hair-like strands or scattered drops. Similarly, the microtubules and microfilaments were either non visible or aggregated in small clusters scattered throughout the ooplasm.

4.5. Discussion.

Our findings demonstrate that calf oocytes can be kept *in vitro* at the germinal vesicle stage for 24 h without affecting their meiotic or developmental competence. Exposure to concentrations of ROS greater than 50 μM prevented meiotic resumption in ~ 60% of oocytes. This meiotic inhibition was fully reversible for oocytes prematured in the presence of 50 μM ROS, since GVBD occurred in all cases and > 70% of oocytes reached the metaphase II stage after a further 24 h of culture in the absence of the inhibitor. Further, these artificially arrested oocytes maintained their developmental competence as indicated by their cleavage rates and blastocyst development following IVM/IVF/IVC.

Table 7: Spindle morphology, chromosome configuration and actin distribution of MII stage calf oocytes after 24 h of culture with different ROS concentrations and an additional 24 h of culture in inhibitor-free medium.

ROS µM	*n**	Spindle configuration, *n* (%)			Chromosome organization, *n* (%)			Actin distribution, *n* (%)			
		Normal	Abnormal	Absent	Dispersed	Decondensed	Absent	Normal	Diffuse	Discontinuous	Absent
Control	56	46 (82.1) a	8 (14.3) a	2 (3.6) a	7 (12.5) a	1 (1.8) a	2 (3.6) a	55 (98.2) a	1 (1.8) a	0 (0) a	0 (0) a
12.5	39	24 (61.5) bc	15 (38.5) b	0 (0) b	13 (33.3) bc	2 (5.1) a	0 (0) b	23 (59.0) b	15 (38.5) b	1 (2.6) b	0 (0) a
25	50	21 (42.0) d	17 (34.0) b	6 (12.0) c	21 (42.0) b	8 (16.0) b	0 (0) b	30 (60.0) b	9 (18.0) c	3 (6.0) b	8 (16.0) b
50	33	25 (75.8) ab	7 (21.2) a	1 (3.0) a	8 (24.2) c	0 (0) c	0 (0) b	32 (97.0) a	0 (0) d	1 (3.0) b	0 (0) a
100	44	23 (52.3) cd	14 (31.8) b	1 (2.3) a	19 (43.2) b	2 (4.5) a	0 (0) b	30 (68.2) b	11 (25.0) c	3 (6.8) b	0 (0) a

* Number of oocytes reaching the MII stage after 24 h of culture with different concentrations of ROS and subsequent culture in inhibitor-free medium for an additional 24 h

Values with different letters within each column are significantly different, $P<0.05$.

Herein, we also demonstrate that the ability of ROS to inhibit the resumption of meiosis is dose-dependent, a concentration of 50 μM being the minimum dose at which ~ 60% of the oocytes are inhibited.

In vivo, the oocyte reaches cytoplasmic maturity (capacitation) and the competence to resume meiosis (maturation) after a long series of processes, in which it is prepared for fertilization and further embryo development. The resumption of meiosis *in vitro* is spontaneously induced by the transfer of meiotically competent oocytes from ovarian follicles into a suitable culture medium. Nevertheless, nuclear meiotic maturation of the oocyte in itself is not enough to ensure subsequent embryo development. Many authors (Lonergan *et al.*, 1997; Avery *et al.*, 1998; Mermillod *et al.*, 2000; Motlik *et al.*, 2000) have tried to mimic the *in vivo* oocyte capacitation process by including a period of prematuration in the presence of meiotic inhibitors. This prematuration interval could enable the oocyte to construct, modify and store newly synthesized proteins and ribonucleoproteins and so enhance its developmental competence.

Several attempts have been made to maintain oocytes at the GV stage, including treatment with cycloheximide (Lonergan *et al.*, 1997; Saeki *et al.*, 1997), 6-dimethylaminopurine (Lonergan *et al.*, 1997; Avery *et al.*, 1998), butyrolactone I (BL-I) (Kitagawa *et al.*, 1993; Motlik *et al.*, 1998; Kubelka *et al.*, 2000; Lonergan *et al.*, 2000), roscovitine (Mermillod *et al.*, 2000), roscovitine plus retinoic acid (Duque *et al.*, 2002) and BL-I plus ROS (Ponderato *et al.*, 2001).

Roscovitine is a selective cdc2 inhibitor, which has been reported to arrest cells in late G1 and at G2/M cell cycle transition. It acts as a competitive inhibitor for ATP and when complexed with cdk2, it binds to the ATP-binding pocket of cdk2**.** Roscovitine has been successfully used to prevent the resumption of meiosis or GVBD in cow (Mermillod *et al.*, 2000; Donnay *et al.*, 2004), pig (Krischek and Meinecke, 2001; Ju *et al.*, 2003; Le Beux *et al.*, 2003; Schoevers *et al.*, 2004) and horse oocytes (Franz *et al.*, 2003).

The extent of meiotic suppression of calf oocytes achieved by ROS in the present study was lower than described for bovine oocytes (88% blockage using 50 μM ROS;

(Mermillod *et al.*, 2000), porcine oocytes (86% using 50 μM ROS; (Krischek and Meinecke, 2001); 81% using 50 μM ROS; (Le Beux *et al.*, 2003); 83% using 80 μM ROS; (Ju *et al.*, 2003)) and equine oocytes (84% using 66 μM ROS; (Franz *et al.*, 2003)).

In general, calf oocytes show reduced developmental competence compared to results obtainable with adult cow oocytes, although this difference has not been clearly established. Indeed, the capacity to form blastocysts at a rate equivalent to that of adult oocytes has been reported for young calves treated with FSH (Armstrong *et al.*, 1992; Irvine *et al.*, 1993; Armstrong *et al.*, 1994). On the contrary, the defective developmental competence of prepubertal oocytes has been suggested by several other studies involving a larger number of animals, a wider age range and the use or lack of use of gonadotropins (Palma, 1994; Looney *et al.*, 1995; Damiani *et al.*, 1996; Duby *et al.*, 1996; Khatir *et al.*, 1996; Presicce *et al.*, 1997). Donnay et al. (2004) reported similar results to ours when they prematured calf oocytes, with 70% of the oocytes remaining at earlier stages to metaphase I after a prematuration treatment using 25 μM ROS and 60% reaching the MII stage after removal of the inhibitor. However, while Mermillod et al. (2000) maintained cattle oocytes at the GV stage for 24 h without decreasing their embryo developmental competence, Donnay et al. (2004) described a considerable decrease in blastocyst rate with respect to non-prematured oocytes (14% vs 4%, control and 25 μM ROS treatments, respectively).

The results obtained in experiment 1 indicate that roscovitine prevented meiotic resumption in around 60% of the oocytes during 24 h of culture at an inhibitor concentration of 50 μM. This concentration is higher than the level of 25 μM used by Mermillod et al. (2000) and Donnay et al. (2004). The factors accounting for this difference are not clear although potential contributing factors are the source and batch of ROS used, source of calf ovaries, the IVM system or the procedure used to assess results. However, the fact that we need a highest ROS concentration (50 μM) to inhibit meiotic resumption of calf oocytes and Salamone et al. (Salamone *et al.*, 2001) reported that the activity of MPF and MAPK was lower in calf oocytes that in oocytes of adult cattle, it could suggest that ROS have also effect over other cytoplasmic factors rather that only the MPF.

In the present study, nuclear and cytoskeletal changes were carefully determined through immunocytochemical staining, defining the three factors: nuclear, microfilament and microtubular status. When GV stage calf oocytes were incubated with roscovitine for 24 h, no effects on spindle formation were observed after IVM in oocytes reaching the MII stage in the presence of 50 µM ROS. However, when oocytes where preincubated with lower (12.5 and 25 µM) or higher (100 µM) concentrations of roscovitine, significantly lower proportions of oocytes showed a normal metaphase spindle after IVM. Moreover, a normal distribution of actin filaments was observed in 85% and 93.2% of oocytes exposed to 50 µM ROS compared to control, respectively. These results are similar to those obtained by Shoevers et al. (2004) in the pig. These authors observed similar proportions of oocytes with normal MII spindles after a prematuration treatment in the presence or absence of ROS. Others, however, have reported chromatin and spindle abnormalities in a substantial proportion of MII stage pig oocytes prematured using a ROS concentration of 80 µM (Ju *et al.*, 2003).

Lonergan et al. (2003) noted morphological changes occurring in immature and *in vitro* matured bovine oocytes following exposure to ROS. These authors demonstrated that a prematuration treatment with 125 µM ROS caused disruption of the integrity of the surrounding cumulus cells, swelling of the mitochondrial cristae and degeneration of the cortical granules. Moreover, convolution of the nuclear membrane and aberrant structures were observed within the nucleoplasm of ROS-treated oocytes. Here, we used ROS concentrations ≤ 100 µM and recorded increases in the percentage of abnormal spindles or abnormal actin distribution at this highest concentration.

In conclusion, our findings indicate the capacity of ROS to maintain calf oocytes in meiotic arrest for 24 h without seriously compromising their potential to develop into blastocysts. The resumption of meiosis and alterations in the chromosome configuration of the meiotic spindle and cytoskeleton distribution were all reversible after removal of the drug.

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CAPÍTULO V:

VITRIFICATION OF CALF OOCYTES: EFFECTS OF MATURATION STAGE AND PREMATURATION TREATMENT ON THE NUCLEAR AND CYTOSKELETAL COMPONENTS OF OOCYTES AND THEIR SUBSEQUENT DEVELOPMENT

5.1. Abstract.

This study was designed to establish the effects of the meiotic stage of bovine oocytes and of a prematuration treatment with roscovitine (ROS) on their resistance to cryopreservation. Calf oocytes at the stages germinal vesicle breakdown (GVBD) and metaphase II (MII) were vitrified by the open pulled straw (OPS) method. In another experiment, GVBD oocytes were prematured with 50 μM ROS before vitrification. After thawing, oocytes in the GVBD and ROS groups underwent an additional 18 h of maturation, while those in the MII group were only subjected to a further 2 h of maturation. After this post-thaw maturation period, some oocyte samples were fixed, stained using specific fluorescent probes and examined under a confocal microscope. The remaining oocytes were fertilized, and cleavage and blastocyst rates recorded. Significantly lower cleavage rates were obtained for the vitrified GVBD and MII oocytes (9.9% and 12.6%, respectively) compared to control oocytes (73.9%). Significantly worse results in terms of cleavage rates were obtained when GVBD calf oocytes were exposed to cryoprotectant (CPA) (13.1%) or vitrified (1.6%) after a prematuration treatment with ROS, when compared to untreated control oocytes (68.7%) or ROS-control oocytes (56.6%). None of the vitrification procedures yielded blastocysts, irrespective of the initial meiotic stage or previous prematuration treatment. Significantly lower proportions of oocytes showing a normal spindle configuration were observed after CPA exposure or vitrification of either GVBD or MII calf oocytes, compared to controls. When GVBD oocytes were vitrified, high percentages of dispersed chromosomes were observed, whereas a prematuration treatment before vitrification increased the proportions of decondensed chromosomes. These results indicate that the vitrification protocol has a deleterious effect on the meiotic spindle organization of calf oocytes cryopreserved at both the GVBD and MII stage, which impairs the capacity for further development of the embryos derived from these vitrified oocytes. Prematuration treatment with ROS has no beneficial effect on the outcome of vitrification by the OPS method.

5.2. Introduction.

Bovine oocytes are much more difficult to cryopreserve than bovine cleavage-stage embryos (Vincent and Johnson, 1992; Aman and Parks, 1994; Martino *et al.*, 1996a;

Martino *et al.*, 1996b; Barnes *et al.*, 1997; Shaw *et al.*, 2000). This sensitive nature of oocytes to cryopreservation may be explained by their unique morphological and functional features, such as their size and the dynamic nature of their subcellular organelles, active biochemical processes and meiosis (Hyttel, 1987; Thibault *et al.*, 1987; Leibfried-Rutledge *et al.*, 1989; Sirard *et al.*, 1989; Niimura and Hosoe, 1995). Although many protocols, such as slow freezing (Lim *et al.*, 1992; Suzuki *et al.*, 1996), rapid cooling (Hochi *et al.*, 1998) or ultrarapid cooling (Martino *et al.*, 1996b) have been attempted, there is no ideal procedure that can produce consistent results. Vajta and co-workers recently developed a vitrification technique for the cryopreservation of bovine oocytes. Using this method, in which the oocytes are deposited in a minimal amount of vitrification solution in open pulled straws (OPS), these authors reported that 25% *in vitro* matured OPS-vitrified bovine oocytes developed to the blastocyst stage after *in vitro* fertilization and culture, and live offspring were produced after the transfer of embryos that were OPS vitrified a second time at the blastocyst stage (Vajta *et al.*, 1998).

Besides the cryopreservation protocol itself, the meiotic stage of the oocytes will also affect the ability of oocytes to survive cryopreservation. Until recently, all efforts were focused on the cryopreservation of mature oocytes, but at this stage, the process induces disorganization of the spindle and the consequent disruption of chromosomes (Magistrini and Szollosi, 1980; Aman and Parks, 1994; Saunders and Parks, 1999) and microfilaments (Saunders and Parks, 1999), an altered distribution of cortical granules (Fuku *et al.*, 1995; Hyttel *et al.*, 2000) and increased polyploidy at fertilization (Glenister *et al.*, 1987; Carroll *et al.*, 1989; Bouquet *et al.*, 1992; Eroglu *et al.*, 1998). Immature oocytes do not have an organized meiotic spindle, and so cryopreservation at this stage may be an alternative approach. Indeed, frozen immature bovine oocytes have given rise to blastocysts (Küchenmeister and Kuwayama, 1997; Le Gal and Massip, 1998), pregnancies (Otoi *et al.*, 1995; Yang *et al.*, 1998) and newborns (Suzuki *et al.*, 1996; Vieira *et al.*, 2002), but rates of *in vitro* embryo development are still very low. This could be explained by the fact that the cells immediately adjacent to the oocyte (the corona radiate) have long cytoplasmic extensions that penetrate the zona pellucida and terminate in bulbous swellings closely associated with the oocyte membrane (Heller *et al.*, 1981; Szollosi *et al.*, 1988). These processes and gap junctions play an important role in the metabolic cooperation that exists between oocytes and cumulus cells during

the growth and final maturation of the oocytes. Some studies have described irreversible structural damage to the oocyte membrane (Arav *et al.*, 1996) or impaired intercellular communication between the oocyte and the cumulus cells after cryopreservation of germinal vesicle (GV) oocytes (Fuku *et al.*, 1995). Therefore, choosing an intermediate stage, such as germinal vesicle breakdown (GVBD), might circumvent some of the problems associated with the cryopreservation of GV or metaphase II (MII) oocytes.

Keeping oocytes in reversible meiotic arrest *in vitro* at the GV stage using a variety of chemicals that interfere with the cAMP transduction pathway has proved feasible in a number of species, particularly in the cow (Mermillod *et al.*, 2000). This so-called prematuration period may provide the necessary time for cytoplasmic maturation to occur, making the oocytes developmentally more competent (Fouladi Nashta *et al.*, 1998). Recently, it has been possible to culture adult and prepubertal calf oocytes under meiotic inhibition without decreasing their developmental potential (Mermillod *et al.*, 2000; Albarracin *et al.*, 2004). The authors involved used roscovitine (ROS), a potent inhibitor of M-phase promoting factor (MPF) kinase activity, for a 24 h culture period before continuing with *in vitro* maturation and fertilization.

In this paper, we describe a set of experiments in which the effects of the oocyte meiotic stage (GVBD vs MII) and the influence of a prematuration treatment with ROS prior to vitrification of GVBD oocytes were evaluated in terms of chromosome, microtubule and microfilament distributions and the ability of the calf oocytes to undergo fertilization and development to the blastocyst stage.

5.3. Materials and methods.

Oocyte collection.

The methods used for the *in vitro* maturation and fertilization of the oocytes have been described elsewhere (Rizos *et al.*, 2001). Briefly, ovaries from slaughtered prepubertal calves were transported from a local abattoir to the laboratory in PBS at 35-37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2- to 10-mm follicles. After three washes in modified PBS (PBS supplemented with 36 µg/mL pyruvate, 50

μg/mL gentamicin and 0.5 mg/mL BSA), groups of up to 50 COCs were placed in 500 μL of maturation medium in four-well plates and cultured for 24 h at 38.5ºC in a 5% CO_2 humidified air atmosphere. The maturation medium was comprised of TCM-199 supplemented with 10% (v:v) fetal calf serum (FCS), 10 ng/mL epidermal growth factor (EGF) and 50 μg/mL gentamicin.

ROS pretreatment.

ROS was prepared as a 5mM stock solution in dimethyl sulfoxide (DMSO), aliquoted and stored at – 20ºC until required. Immature oocytes were incubated in TCM-199 supplemented with 10% (v:v) FCS, 10 ng/mL EGF, 50 μg/mL gentamicin and 50 μM ROS for 24 h at 38.5ºC under an atmosphere of 5% CO_2 in air with maximum humidity. After the incubation period, the oocytes were rinsed several times in modified PBS to avoid carry-over of ROS into the oocyte maturation medium and then cultured in maturation medium for a further 6 h at 38.5ºC in a 5% CO_2 humidified air atmosphere.

In vitro fertilization.

For *in vitro* fertilization, the COCs were washed four times in PBS and then in the fertilization medium before being transferred, in groups of up to 50, to four-well plates containing 250 μL of fertilization medium per well (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 μg/mL heparin-sodium salt). Motile spermatozoa were obtained by centrifuging frozen-thawed sperm from Asturian bulls on a discontinuous Percoll density gradient (2.5 mL 45% Percoll over 2.5 mL 90% Percoll) for 8 min at 700 x g at room temperature. Viable spermatozoa, collected from the bottom of the 90% fraction, were washed in Hepes-buffered Tyrode's and pelleted by centrifugation at 100 x g for 5 min. Spermatozoa were counted in a hemocytometer and diluted in an appropriate volume of fertilization medium to give a final concentration of 2×10^6 spermatozoa/mL. A 250-μL aliquot of this suspension was then added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/mL. Plates were incubated for 24 h at 38.5ºC in a 5% CO_2 humidified air atmosphere. Semen from the same bulls was used in all the experiments.

In vitro development.

At approximately 22 h post-insemination (hpi), presumptive zygotes were denuded by gentle vortexing and washed four times in PBS and twice in the culture medium before being transferred to 25 µl culture droplets (1 embryo/µl) under mineral oil. Culture was conducted in synthetic oviductal fluid (SOF) (Holm *et al.*, 1999) for 8 days at 38.5ºC in a 5% CO_2, 5% O_2, 90% N_2 humidified atmosphere. Cleavage rates were recorded at 48 hpi and numbers of blastocysts were determined on post-insemination Days 7, 8 and 9.

Oocyte vitrification.

The vitrification procedure was essentially as described by Vajta et al. (1998). All manipulations were performed on a 41ºC hot plate in a room at 25-27ºC. Six hours (for GVBD stage oocytes) or 22 h (for MII stage oocytes) after the onset of maturation, oocytes were denuded by gently pipetting in PBS until only two to four layers of cumulus cells remained on the surface. Oocytes were first equilibrated in a holding medium (TCM199-HEPES supplemented with 20% FCS) for 5 min and initially dehydrated by 30 s exposure to 1 mL 10% ethylene glycol (EG) + 10% DMSO in TCM199-HEPES + 20% FCS. The oocytes were then transferred to 20-µL droplets of 20% EG + 20% DMSO in TCM199-HEPES + 20% FCS + 0.5 M sucrose. Straws were loaded with three to five oocytes each, by touching the surface of a 1-1.5-µL droplet of vitrification solution containing the oocytes with the narrow tip of the OPS. The loaded straw was then directly plunged into liquid nitrogen within 25 s. Warming was performed by exposing the OPS straw to air for 3 s prior to directly immersing the narrow tip of the straw into 0.8 mL of TCM199-HEPES medium containing 20% FCS + 0.25 M sucrose. The oocytes were directly expelled into the medium after the vitrified medium became liquid. After about 5 min, the oocytes were transferred to 0.15 M sucrose for further rehydration. They were then washed in 1.2 mL holding medium for 5 min and *in vitro* maturation continued in the original dish for 18 (GVBD) or 2 (MII) additional hours.

Experiment 1

In this experiment, calf oocytes were exposed to CPA (EG+DMSO), or vitrified at the GVBD or MII stage. Oocytes were randomly assigned to one of 5 experimental groups: (1) control, oocytes matured *in vitro* for 24 h; (2) oocytes matured for 6 h (GVBD stage) and exposed to different concentrations of CPA without vitrification; (3) oocytes matured for 6 h (GVBD stage) and vitrified by the OPS method; (4) oocytes matured for 22 h (MII stage) and exposed to different concentrations of CPA without vitrification; (5) oocytes matured for 22 h (MII stage) and vitrified by the OPS method. Oocytes in groups 2 and 3 were *in vitro* matured for an additional 18 hours and those in groups 4 and 5 matured for 2 further hours. The experiment was repeated four times.

After 24 h of *in vitro* maturation, a sample of oocytes from each experimental group was fixed and evaluated using specific fluorescent probes before visualization using a laser-scanning confocal microscope. The remaining oocytes were fertilized and cleavage rates were recorded.

Experiment 2

In this experiment, calf oocytes were treated with 50 μM ROS for 24 h and subsequently exposed to CPA or vitrified by the OPS method at the GVBD stage. Oocytes were randomly assigned to one of 6 experimental groups: (1) control oocytes matured *in vitro* for 24 h; (2) oocytes matured for 6 h and exposed to different concentrations of CPA without vitrification; (3) oocytes matured for 6 h and vitrified by the OPS method; (4) oocytes prematured for 24 h in the presence of 50 μM ROS and then matured *in vitro* for 24 additional hours; (5) oocytes prematured for 24 h in the presence of 50 μM ROS, matured for 6 h and exposed to different concentrations of CPA without vitrification; (6) oocytes prematured for 24 h in the presence of 50 μM ROS, matured for 6 h and vitrified by the OPS method. Oocytes in experimental groups 2, 3, 5 and 6 were subjected to a further 18 hours of *in vitro* maturation. The experiment was repeated four times.

After 24 h of *in vitro* maturation, a sample of oocytes from each experimental group was fixed and evaluated using specific fluorescent probes before visualization using a laser-scanning confocal microscope. The remaining oocytes were fertilized and cleavage rates were recorded.

Oocyte immunostaining.

Samples of oocytes from the experimental groups (see above) were fixed in 2% formaldehyde PBS, permeabilized using Triton X-100 (0.2% in PBS) and simultaneously immunostained for actin filaments, tubulin and chromatin detection (Boiso *et al.*, 2002). Fixation and subsequent incubations were performed at 37ºC.

For immunostaining, fixed oocytes were incubated with the anti-α-tubulin monoclonal antibody (1:250) for 1.5 h, followed by incubation with anti-mouse IgG antibody-biotin (1:5000) for 1 h, avidin-Cy5 for 30 min and falloidin-fluorescein isothiocyanate (FICT) conjugate (1:1000) for 1 h. Between incubations, the oocytes were washed three times in pre-warmed PBS for 5 min.

Groups of five oocytes were mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and then stained with DAPI (125 ng/mL). The preparation was sealed with nail varnish and stored refrigerated and protected from light until observation within the following two days. A laser-scanning confocal microscope (Leica TCS-SP2-AOBS) was used to examine actin filaments (FICT; excitation 488 nm), tubulin (Cy5; excitation 633 nm) and chromatin (DAPI; excitation 405 nm). Images were recorded on a computer.

Figure 1 shows the normal nuclear and cytoskeletal morphology of calf oocytes at the stages GV, GVBD, MI and MII. In oocytes at the metaphase stage of the second meiotic division, the spindle structure always appears at the periphery, oriented perpendicularly to the plasma membrane. Oocytes that reached the MII stage were classified as follows:

A) The spindle morphology of MII oocytes was classified as three categories: (1) *Normal spindles*. These were barrel-shaped with chromosomes clustered as a discrete bundle at the metaphase plate and microtubules crossing the length of the spindle from

pole to pole, or extending from the spindle poles to the chromosomes; microtubules within the polar body had no discernable organization and instead appeared as an amorphous mass intertwined with chromatin occupying the perivitelline space; (2) *Abnormal spindles*. Microtubules were not organized as typical spindles or some microtubules were disassembled; (3) *Absence of the spindle*. No microtubules could be observed around the chromosomes. Detailed images of these normal and abnormal patterns are provided in Figure 1.

B) Chromosomal organization was classified as: (1) *Dispersed chromosomes*. These were scattered in the cytoplasm or dispersed in a few zones of the cytoplasm; (2) *Decondensed chromosomes*. Chromosomes with an aberrant, less condensed appearance; (3) *Absence of chromosomes*. No chromosomes were observed (Figure 1).

C) The cytoskeleton actin band in the MII oocytes was classified as four categories: 1) *Normal*. An evenly stained layer of actin appearing immediately beneath the plasma membrane; 2) *Diffuse*. Diffuse, irregular microfilament distribution, weakly stained; 3) *Discontinuous*. Interrupted staining of the actin band; 4) *Absence of actin band*. No microfilaments observed beneath the oolema (Figure 1).

Statistical analysis.

The χ^2-test was used to compare data among the experimental groups. The level of statistical significance was set at $P < 0.05$.

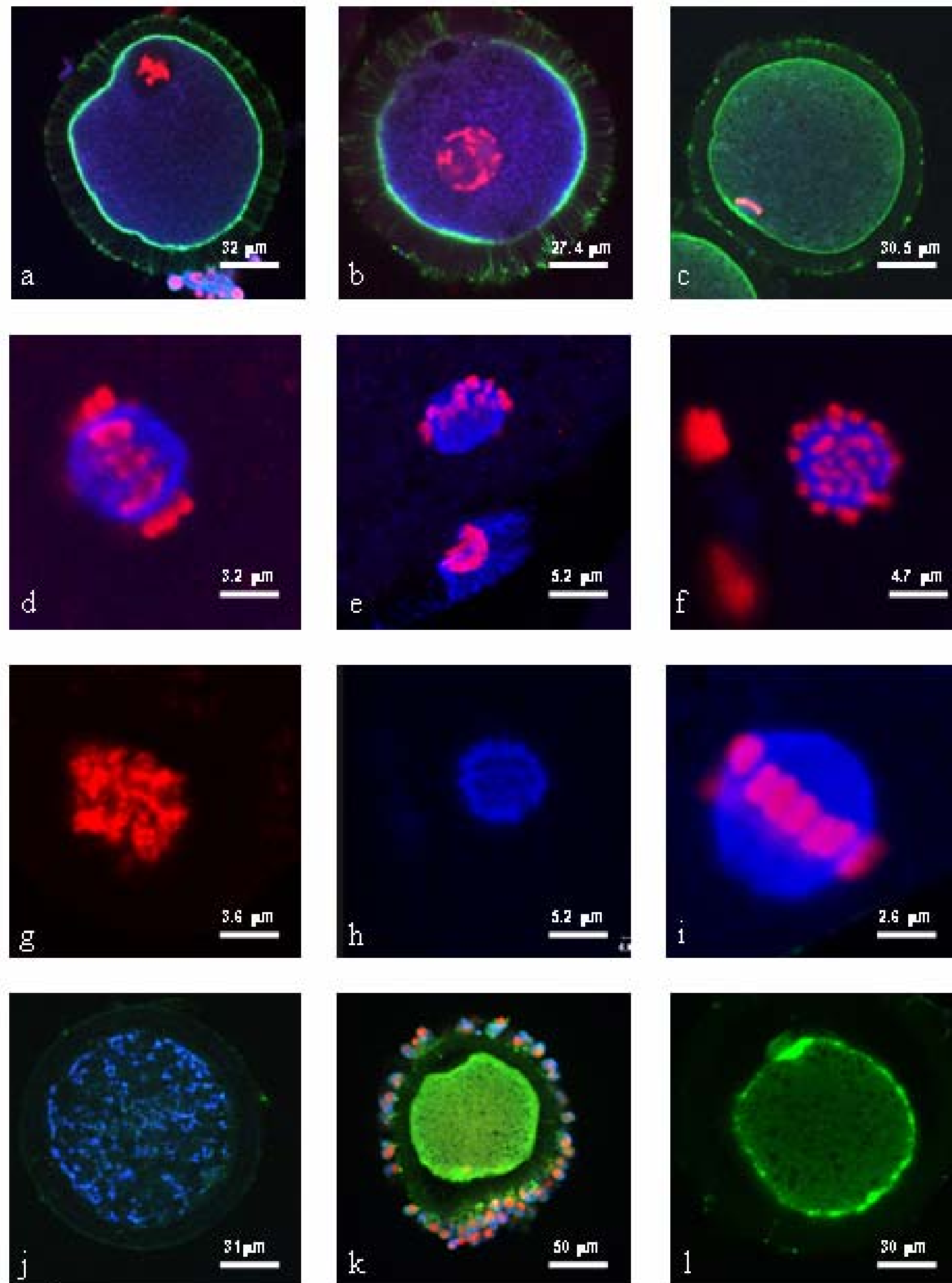

Figure 1. Confocal laser-scanning photomicrographs of calf oocytes at different stages of maturation *in vitro* (a-d). Alterations in the spindle morphology, chromosome configuration and microfilament distribution of calf MII oocytes (e-l). Oocytes were immunocytochemically stained using an anti-α-tubulin monoclonal antibody and avidin-Cy5 to visualize the microtubules (blue), counterstained with DAPI to visualize chromosomes (red) and with phalloidine-fluorescein isothiocyanate to visualize actin filaments (green). (a) Image of a germinal vesicle stage oocyte. Note the chromatin has yet to condense, and microtubules form a network throughout the ooplasm. Microfilaments appear just beneath the plasma membrane of the oocyte. (b) A germinal vesicle oocyte at the breakdown stage. Note the microtubule asters associated with the now condensed chromatin strands, while the microfilaments clearly concentrate inside the oocyte cortex. (c) Metaphase II stage oocyte with microtubules having now formed a clear meiotic spindle with chromosomes aligned at its equator. (d) Normal barrel-shaped metaphase II spindle with compact chromosomes arranged at the equator of the structure. Microtubules can be seen within the spindle. (e) Abnormal spindle structures associated with partly disorganized chromosomes. The polar body can be observed. (f) Abnormal spindle structure associated with completely disorganized microtubules and chromosomes. (g) Chromosomes with an aberrant, less condensed appearance. Note the absence of microtubules. (h) Disrupted microtubular shape. (i) Slightly abnormal spindle structure with rounded poles. (j) Cytoplasmic microtubule asters formed in oocytes treated with 50 µM ROS and vitrified by the OPS technique. No actin band staining can be observed. (k) Oocytes showing a diffuse actin band. (l) Discontinuous distribution of microfilaments.

5.4. Results.

Experiment 1

Oocytes at the GVBD stage were compared to those at the MII stage (Table 1). Exposure to CPA or the vitrifying-warming process significantly reduced the developmental capacity of oocytes compared to controls. Similar cleavage rates were obtained from oocytes in both the GVBD and MII groups when these were exposed to CPA (39.5% and 48.9% for GVBD and MII oocytes, respectively) or vitrified (9.9% and 12.6% for GVBD and MII oocytes, respectively). However, blastocyst rates were null for calf oocytes vitrified in OPS at either the GVBD or MII stage, or exposed to CPA at the GVBD stage.

Table 1: Effect of calf oocyte maturity on developmental competence after CPA exposure or OPS vitrification (Experiment 1).

	n (%) of oocytes		
	Treated	Cleaved	Developed to blastocysts
Control	173	128 (73.9) a	13 (10.1) a
GVBD-CPA	114	45 (39.5) b	0 (0) b
GVBD-OPS	121	12 (9.9) c	0 (0) b
MII-CPA	135	66 (48.9) b	10 (7.4) a
MII-OPS	119	15 (12.6) c	0 (0) b

Values with different letters within each column are significantly different, $P<0.05$

The results of the analysis of spindle, chromosome and microfilament organization are shown in Table 2. Significantly higher proportions of oocytes, both at the GBVD and MII stage, with abnormal spindle configuration were observed after exposure to CPA or vitrification compared to control. After exposure to CPA of GVBD or MII stage oocytes, similar percentages of oocytes showing normal spindle patterns were obtained. Similar effect was observed after vitrifying GVBD or MII stage oocytes. When GVBD oocytes were vitrified, a high percentage had dispersed chromosomes (66.7%) while vitrification of MII oocytes mainly provoked chromosome dispersion and decondensation (37.5% and 25.0%, respectively).

Table 2. Spindle morphology, chromosome configuration and actin distribution of GVBD and MII calf oocytes exposed to CPA or vitrified by the OPS method (Experiment 1).

	n	MII, *n* (%)	Spindle configuration, *n* (%*)			Chromosome organization, *n* (%*)			Actin distribution, *n* (%*)			
			Normal	Abnormal	Absent	Dispersed	Decondensed	Absent	Normal	Diffuse	Discontinuous	Absent
Control	20	17 (85.9)a	15 (88.2)a	1 (5.9)a	1 (5.9)a	1 (5.9)a	0 (0)a	1 (5.9)a	16 (94.1)a	1 (5.9)a	0 (0)a	0 (0)a
GVBD-CPA	32	19 (59.4)bc	8 (42.1)bd	7 (36.8)bc	4 (21.1)b	6 (31.6)bd	5 (26.3)b	0 (0)b	16 (84.2)b	1 (5.3)a	1 (5.3)b	1 (5.3)b
GVBD-OPS	33	15 (45.5)b	4 (26.7)c	11 (73.3)d	0 (0)c	10 (66.7)c	0 (0)a	1 (6.7)a	11 (73.3)bc	1 (6.7)a	3 (20.0)c	0 (0)a
MII-CPA	24	20 (83.3)a	11 (55.0)d	6 (30.0)b	3 (15.0)b	5 (25.0)b	3 (15.0)b	1 (5.0)a	15 (75.0)bc	4 (20.0)b	1 (5.0)b	0 (0)a
MII-OPS	26	16 (61.5)c	6 (37.5)bc	7 (43.8)c	3 (18.8)b	6 (37.5)d	4 (25.0)b	0 (0)b	11 (68.8)c	1 (6.3)a	3 (18.8)c	1 (6.3)b

*: Percentage referred to the total number of oocytes reaching the MII stage

Values with different letters within each column are significantly different, P<0.05.

In most untreated oocytes (94.1%), actin showed a compact and homogeneous staining pattern around the oolema periphery. Exposure of MII oocytes to CPA significantly increased the percentages of oocytes with a diffuse actin band. The proportion of oocytes with a discontinuous actin pattern was significantly increased after CPA exposure, and this increase was significantly greater after vitrification, irrespective of the meiotic stage.

Experiment 2

Table 3 shows the effect of a prematuration treatment with ROS (50µM) on the developmental competence of calf oocytes after CPA exposure or OPS vitrification at the GVBD stage. Exposure to CPA or vitrification of GVBD oocytes significantly decreased cleavage rates with respect to controls (36.3% and 10.6% compared to 68.7%, respectively). When GVBD calf oocytes were exposed to CPA (13.1%) or vitrified (1.6%) after a prematuration treatment with ROS, significantly worse cleavage rates were obtained when compared to control oocytes (68.7%) or to ROS-control oocytes (56.6%). When embryos were cultured to the blastocyst stage, no blastocysts were obtained after exposure to CPA or vitrification, irrespective of whether they had been prematured or not with ROS.

Table 3: Effect of a prematuration treatment with ROS (50µM) on the developmental competence of GVBD calf oocytes after CPA exposure or OPS vitrification (Experiment 2).

	n (%) of oocytes		
	Treated	Cleaved	Developed to blastocysts
Control	115	79 (68.7) a	12 (10.4) a
CPA	102	37 (36.3) b	0 (0) b
OPS	113	12 (10.6) c	0 (0) b
ROS-Control	113	64 (56.6) a	7 (6.2) a
ROS+CPA	115	15 (13.1) c	0 (0) b
ROS+OPS	128	2 (1.6) d	0 (0) b

Values with different letters within each column are significantly different, $P<0.05$

Table 4 shows a summary of the observations made of the spindles. The maturation rate achieved was significantly higher for control oocytes (88.9%) with respect to GVBD oocytes exposed to CPA (60.0%) or vitrified (45.5%). When GVBD calf oocytes were vitrified (27.9%) after prematuration treatment with ROS, significantly lower maturation rates were observed compared to ROS-control oocytes (75.5%). The maturation rates of oocytes exposed to CPA after prematuration (63.6%) did not differ significantly from those recorded for ROS-control oocytes (75.5%). Normal spindle structures were observed in 90.0% of the control oocytes, whereas this percentage decreased for GVBD oocytes exposed to CPA (40.0%) or vitrified (25%). Prematuration treatment with ROS did not significantly improve the percentage of normal spindles observed, with values of 45.7% or 16.7% recorded after CPA exposure or OPS vitrification, respectively.

When GVBD oocytes were exposed to CPA or vitrified, the microtubules in the spindle of most oocytes appeared not to be organized in a defined spindle shape or were partially or completely disassembled. When oocytes were exposed to CPA, with or without prematuration treatment, the proportions of abnormal or missing spindles increased significantly with respect to control oocytes. When GVBD oocytes were vitrified by the OPS technique, significantly higher percentages of abnormal microtubule patterns were observed, either with or without prematuration. However, a novel abnormal microtubular pattern was observed when oocytes were pretreated with ROS and then OPS vitrified. Six oocytes (out of 12) showed numerous microtubular asters and a microtubular network formed by radiating arrays of these asters, as shown in Figure 1J.

On the other hand, the proportions of oocytes with dispersed chromosomes in the cytoplasm increased significantly after all the treatments tested. The highest percentage of oocytes with dispersed chromosomes was observed in the group of GVBD oocytes that had been vitrified (65.0%), while oocytes prematured with ROS and vitrified yielded the highest proportions with decondensed chromosomes (50.0%).

Oocytes vitrified either with or without a prematuration treatment showed significantly lower percentages of normal microfilament patterns while CPA exposure did not modify actin distribution when compared to control oocytes.

Table 4: Spindle morphology, chromosome configuration and actin distribution of GVBD calf oocytes pretreated with ROS (50μM) and exposed to CPA or vitrified in OPS (Experiment 2).

	n	MII, *n* (%)	Spindle configuration, *n* (%*)			Chromosome organization, *n* (%*)			Actin distribution, *n* (%*)			
			Normal	Abnormal	Absent	Dispersed	Decondensed	Absent	Normal	Diffuse	Discontinuous	Absent
Control	45	40 (88.9)a	36 (90.0)a	2 (5.0)a	2 (5.0)a	2 (5.0)a	0 (0)a	2 (5.0)a	38 (95.0)a	2 (5.0)	0 (0)a	0 (0)a
CPA	50	30 (60.0)b	12 (40.0)b	10 (33.3)b	8 (26.7)b	13 (43.3)bc	3 (10.0)b	2 (6.7)a	28 (93.3)a	1 (3.3)	1 (3.3)bc	0 (0)a
OPS	44	20 (45.5)c	5 (25.0)c	15 (75.0)c	0 (0)b	13 (65.0)d	2 (10.0)b	0 (0)b	16 (80.0)b	1 (5.0)	2 (10.0)b	1 (5.0)b
ROS-Control	45	34 (75.5)ad	26 (76.4)d	6 (17.6)d	2 (5.9)a	7 (20.5)e	1 (2.9)c	0 (0) b	31 (91.1)a	1 (2.9)	2 (5.9)bc	0 (0)a
ROS+ CPA	55	35 (63.6)bd	16 (45.7)b	16 (45.7)b	3 (8.6)a	18 (51.4)b	0 (0)a	1 (2.9)a	33 (94.3)a	1 (2.9)	1 (2.9)c	0 (0)a
ROS+ OPS	43	12 (27.9)e	2 (16.7)c	10 (83.3)c	0 (0)b	4 (33.3)c	6 (50.0)d	0 (0)b	9 (75.0)b	1 (8.3)	1 (8.3)bc	1 (8.3)b

*: Percentage calculated over the total number of oocytes reaching the MII stage

Values with different letters within each column are significantly different, $P<0.05$.

5.5. Discussion.

The results of Experiment 1 indicate that the meiotic stage has no effect on the ability of calf oocytes to survive cryopreservation, with both GVBD and MII stage oocytes being similarly able to withstand the cryopreservation process. Further, handling control oocytes, which were only exposed to CPA without further cooling, showed a reduced capacity for development compared to untreated controls, suggesting that osmotic shock compromises the success of cryopreservation procedures.

There have been a number of attempts over the last few decades to cryopreserve oocytes at various stages (Lim *et al.*, 1992; Otoi *et al.*, 1995; Eroglu *et al.*, 1998; Hochi *et al.*, 1998; Men *et al.*, 2002). These investigations have established that oocytes at different stages show differential responses to temperature shock. In fact, several authors have reported that GV oocytes are more sensitive to chilling or cryopreservation than MII oocytes (Lim *et al.*, 1992; Fuku *et al.*, 1995; Zeron *et al.*, 1999; Goud *et al.*, 2000). Bovine oocytes at the GVBD stage have been described as more resistant to cooling than GV or MII oocytes (Barnes *et al.*, 1997). However, when cryopreservation was attempted, Men at al. (2002) observed that a significantly higher proportion of cleaved bovine embryos from vitrified MII oocytes developed into blastocysts than those derived from vitrified GVBD oocytes. In addition to GVBD oocytes, attempts have also been made at vitrifying oocytes at other maturation stages. Thus, when bovine oocytes were vitrified 0, 6, 12 or 24 h after the onset of maturation, Hochi et al. (1998) found the best stage for vitrification was that of oocytes matured for 12 h. Le Gal and Massip (1999) reported similar percentages of blastocysts following the vitrification of bovine oocytes at 0, 17 or 24 h after the start of the maturation process.

In the present study, nuclear and cytoskeletal changes were carefully determined through immunocytochemical staining, defining the three factors: nuclear, microfilament and microtubular status. When calf oocytes were exposed to CPA or vitrified either at the GVBD or MII stage, they showed significantly lower percentages of both normal spindle and chromosome configurations than untreated oocytes. Temperature fluctuations are known to directly affect the cytoskeletal organization and chromosome configuration of mouse (Pickering and Johnson, 1987), human (Pickering *et al.*, 1990; Almeida and Bolton, 1995), bovine (Richardson and Parks, 1992; Aman

and Parks, 1994; Saunders and Parks, 1999; Wu *et al.*, 1999) and porcine (Liu *et al.*, 2003; Rojas *et al.*, 2005) oocytes. The partial or complete disassembly of microtubules in the spindle has been observed after cooling oocytes to room temperature or lower (Pickering and Johnson, 1987; Pickering *et al.*, 1990; Aman and Parks, 1994; Almeida and Bolton, 1995). When returned to normal temperature, the spindle recovers although there are species-specific differences. The spindles of mouse oocytes undergo complete disassembly after a 45 min to 60 min period at 0ºC, but the spindles of most oocytes reassemble after rewarming (Magistrini and Szollosi, 1980). However, the spindles of human (Almeida and Bolton, 1995; Wang *et al.*, 2001), cow (Richardson and Parks, 1992; Aman and Parks, 1994), sheep (Moor and Crosby, 1985) or pig (Liu *et al.*, 2003) oocytes only undergo limited recovery after cooling-rewarming treatment. The meiotic spindle becomes completely disassembled when *in vitro* matured bovine oocytes are maintained for 10 to 20 min at 4ºC (Richardson and Parks, 1992). Similar findings were reported by Wu et al. (1999), who noted a drastic decrease in the formation of normal meiotic spindles when immature bovine oocytes were chilled to 4ºC for as little as 10 min. The fact that cooling immature oocytes, with no microtubular spindle present, affects subsequent meiotic spindle formation and polar body extrusion implies that cooling may lead to the damage of key regulatory factors, such as MPF, mitogen-activated protein kinase (MAPK) or others, affecting microtubule organization (Vincent and Johnson, 1992; Wu *et al.*, 1996; Wu *et al.*, 1997).

Previous studies have shown that cryoprotectants disrupt the cortical microfilament network and cause depolymerization and disorganization of the spindle microtubules, which in turn results in chromosomal scattering (Magistrini and Szollosi, 1980; Jonhson and Pickering, 1987; Vincent *et al.*, 1989; Vincent *et al.*, 1990). EG may affect the dynamic assembly and disassembly of microtubules, as has been shown for DMSO or propanediol (Vincent *et al.*, 1989). At room temperature, the addition of cryoprotectant (propanediol or DMSO) to rabbit oocytes leads to the disorganization of microtubule and microfilament distribution (Vincent *et al.*, 1989). In mouse oocytes, a similar effect of DMSO (Johnson and Pickering, 1987; Van der Elst *et al.*, 1988) and propanediol (Van der Elst *et al.*, 1988) has also been noted and the consequent dispersal of chromosomes is often seen in both species. The exposure of bovine oocytes to 1.5M EG for 10 min has been observed to increase abnormal spindle formation, although this treatment did not affect normal microfilament distribution (Saunders and Parks, 1999).

The factors likely to contribute to the different sensitivity of immature oocytes compared to mature oocytes are the specific structure of the zona pellucida, the cytoskeleton, the cytoplasmic membranes and their composition (Sathananthan, 1994; Ji *et al.*, 1997) along with the connections of cumulus-cell projections and the oocyte cytoskeleton through their junctions with the oolema (Allworth and Albertini, 1993). It is important that these connections remain intact for the completion of normal cytoplasmic maturation *in vitro*. In bovine oocytes, active transcription and translation occur at the GVBD and later stages of meiotic maturation (Hunter and Moor, 1987; Leibfried-Rutledge *et al.*, 1989; Sirard *et al.*, 1989). Therefore, in addition to detrimental effects on cytology, cryopreservation may also disrupt the biochemical processes occurring within the oocytes with the consequent negative effects on cytoplasmic maturation.

Some authors report a loss of cumulus cells from the COC following cryopreservation and thawing (Van der Elst *et al.*, 1993; Cooper *et al.*, 1998; Goud *et al.*, 2000). By examining the ultrastructure of immature bovine (Fuku *et al.*, 1995) and equine (Hochi *et al.*, 1996) oocytes after vitrification, some have suggested that freezing damage is associated with the destruction of these intercellular contacts between cumulus cells and the oocyte. The three-dimensional COC is likely to be particularly prone to the physical disruption caused by ice crystal formation. Even when ice crystal formation is avoided during vitrification, the vast difference in size between the oocyte and its associated cumulus cells means that they are likely to react very differently to the stresses in play during cryopreservation. Ruppert-Lingham et al. (2003) tried to overcome this problem by co-culturing cryopreserved mouse GV oocytes with fresh disassociated cumulus cells, although no improvement on embryo development was observed.

Men et al. (2002) achieved blastocysts after vitrifying GVBD and MII cow oocytes by the OPS method. However, we were unable to obtain blastocysts from calf oocytes vitrified either at the GVBD or MII stage. Our previous studies described in Chapter III revealed that calf oocytes were more sensitive to freezing injury than adult cow oocytes, yielding lower percentages of cleavage and blastocyst formation after vitrification. Oocytes collected form prepubertal animals are known to be less competent after *in vitro* maturation, fertilization and embryo culture than oocytes collected from adult animals (Revel *et al.*, 1995; Damiani *et al.*, 1996; Duby *et al.*, 1996; Khatir *et al.*, 1996;

Presicce *et al.*, 1997; Taneja *et al.*, 2000). This deficient developmental capacity of calf oocytes is most probably attributable to their abnormal cytoplasmic maturation (Salamone *et al.*, 2001).

Several authors have hypothesized that if oocytes are cultured before maturation *in vitro* under conditions that arrest meiosis at the GV stage, this might offer them the opportunity to acquire greater developmental competence (Lonergan *et al.*, 2000; Mermillod *et al.*, 2000). In the present study, we subjected calf oocytes to a prematuration treatment with ROS before vitrification, in an effort to improve oocyte cytoplasmic maturation and increase the developmental capacity of the thawed oocytes. Our results, however, indicate no such improvement and lower cleavage rates were obtained after CPA exposure or vitrification of prematured oocytes compared to non-prematured oocytes. On the other hand, similar percentages of oocytes showing a normal spindle and cytoskeleton configuration were observed after CPA exposure or vitrification, irrespective of the prematuration treatment. Morató et al. (2004) reported similar proportions of oocytes with normal MII spindles for non-prematured oocytes and those treated with 50 µM ROS. In contrast, Lonergan et al. (2003) described the disruption of the integrity of the surrounding cumulus cells, swelling of the mitochondrial cristae and degeneration of cortical granules in ROS-prematured bovine oocytes. In addition to the possible detrimental effects of prematuration treatment, the vitrification process is known to induce intense ultrastructural modifications to the microvilli, mitochondria, vesicle formation and the ooplasm of immature bovine oocytes (Fuku *et al.*, 1995). Hence, although ROS prematuration treatment failed to alter normal spindle and actin configurations, our significantly lowered cleavage rates might have been a consequence of both the ultrastructural changes induced by ROS and the detrimental effects of crypreservation.

Collectively, our findings suggest that the vitrification protocol used has deleterious effects on the organization of the meiotic spindle of calf oocytes cryopreserved at both the GVBD and MII stage, impairing the capacity for further development of the embryos derived from these vitrified oocytes. A prematuration treatment with ROS had no beneficial effect on oocytes vitrified by the OPS method. The cryoinjury suffered by the oocytes was the main determinant for the low cleavage rates and poor

developmental potential of these frozen oocytes. Further research will help clarify the cellular and molecular mechanisms of cryopreservation-induced injury.

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CAPÍTULO VI:
DISCUSIÓN GENERAL.

El primer capítulo experimental de esta tesis doctoral se centró en el estudio de los efectos de la técnica de vitrificación mediante OPS sobre las estructuras de la placa metafásica, del citoesqueleto y el desarrollo embrionario en ovocitos de vacas prepúberes y adultas. En este estudio, se observó que tanto la exposición a los crioprotectores como el proceso de vitrificación provocaba daños a nivel estructural y por tanto, una disminución en los porcentajes de ovocitos que llegaban al estadio de blastocisto, tanto en ovocitos procedentes de ovarios de vaca como de ternera. Además, se determinó que los ovocitos de ternera son más resistentes a la exposición a las soluciones crioprotectoras pero más sensibles a la vitrificación puesto que se observó una disminución significativa del porcentaje de ovocitos de ternera que presentaban husos y/o distribuciones de actina normales en comparación con los ovocitos procedentes de ovarios de vaca. Asimismo, el desarrollo embrionario *in vitro* de los ovocitos de ternera tras su vitrificación y fecundación fue inferior al de los ovocitos de vaca. La técnica de OPS desarrollada por Vajta *et al*. (1998) para la vitrificación de ovocitos bovinos madurados *in vitro* es la que mejores porcentajes de desarrollo embrionario obtiene comparada con otras técnicas de vitrificación para el mismo estadio de maduración, utilizando diferentes soportes y/o crioprotectores (Martino *et al.*, 1996; Le Gal y Massip, 1999; Rho *et al.*, 2002). Estos resultados nos llevaron a pensar que esta técnica sería la más adecuada para llevar a cabo la vitrificación de ovocitos procedentes de terneras. En nuestro laboratorio, el porcentaje de blastocistos obtenido tras vitrificar ovocitos de vaca y ternera mediante esta técnica fue inferior al alcanzado Vajta *et al.* (1998), pero similar al obtenido por otros autores (Le Gal y Massip, 1999).

Nuestros resultados confirmaron el hecho de que los ovocitos de ternera, aún presentando una división celular similar a la ovocitos adultos, poseen una menor capacidad de desarrollo embrionario. Este hecho es explicado por varios autores como una deficiencia o retraso en la capacidad de maduración citoplasmática de los ovocitos procedentes de animales prepúberes (Khatir *et al.*, 1998; Mermillod *et al.*, 1998; Armstrong, 2001; Salamone *et al.*, 2001).

Los resultados de desarrollo embrionario obtenidos en este estudio indicaron que los ovocitos de ternera poseen una capacidad similar a los ovocitos procedentes de animales adultos para resistir el daño osmótico causado por los crioprotectores. Sin embargo, estos mismos ovocitos son más susceptibles al daño causado por la congelación

comparado con sus similares procedentes de adulto. Minimizar el daño osmótico es un punto importante en las técnicas de vitrificación, ya que, para conseguir velocidades de enfriamiento lo suficientemente altas para evitar la formación de cristales de hielo, es necesario exponer al ovocito a concentraciones multimolares del crioprotector (revisado por Shaw *et al.*, 2000; Vajta, 2000). Ello provoca que el ovocito sufra alteraciones estructurales derivadas de este cambio osmótico, expresadas en el cambio de volumen que sufre la célula en contacto con el crioprotector hiperosmótico y que afecta al citoesqueleto del ovocito.

Diversos autores sugieren que el principal objetivo a desarrollar en los protocolos de crioconservación es el de tratar de preservar la integridad de la placa metafásica durante el enfriamiento (Eroglu *et al.*, 1998). La principal consecuencia del enfriamiento sobre la placa metafásica es la despolimerización y la desaparición de los microtúbulos (Webb *et al.*, 1986). El reensamblaje de esta estructura es posible una vez es restituida la temperatura normal (Inoue, 1981), aunque no siempre se da de manera correcta, observándose que un porcentaje de estas estructuras puede presentar alteraciones en los patrones de distribución de los cromosomas o los microtúbulos (Richardson y Parks, 1992; Liu *et al.*, 2003). La exposición de los ovocitos maduros de distintas especies como el ratón (Vincent y Johnson, 1992; Cooper *et al.*, 1996), la coneja (Vincent *et al.*, 1989), la mujer (Sathananthan *et al.*, 1987) y la vaca (Saunders y Parks, 1999) a los crioprotectores también presentó un efecto adverso sobre la estructura de los microtúbulos. De hecho, el contacto del propanodiol o el DMSO con ovocitos de conejo madurados *in vitro* provocó la desorganización de los microtúbulos de la placa metafásica (Vincent *et al.*, 1989). Similar efecto fue observado en ovocitos de ratón expuestos al propanodiol (Van der Elst *et al.*, 1988).

La disrupción del citoesqueleto constituye un efecto intrínseco a los cambios de volumen que sufre el ovocito como consecuencia de los procedimientos de crioconservación. Sin embargo, en este estudio no se observó que la exposición de los ovocitos de ternera y vaca a concentraciones multimolares de crioprotector tuviera un efecto significativo sobre la disrupción de los filamentos de actina. Williams et al. (1992) observaron que la organización de los microfilamentos de ovocitos bovinos expuestos a EG o a propanodiol fue parecida a la de los ovocitos control. Saunders y Parks (1999) evidenciaron la presencia de bandas normales de distribución de la actina

después de 1-3 horas post-incubación de ovocitos bovinos que habían sido expuestos a EG.

Por otra parte, el proceso de vitrificación por OPS llevado a cabo en este estudio provocó una disminución significativa de los ovocitos que presentaban un patrón de distribución normal de los filamentos de actina, tanto en ovocitos procedentes de vaca como de ternera. Similares efectos fueron observados en ovocitos de ratón y conejo (Vincent *et al.*, 1989; George y Johnson, 1993) aunque estos cambios fueron reversibles tras la descongelación. Por otra parte, la congelación lenta de ovocitos maduros de vaca comportó una disminución significativa en el porcentaje de ovocitos con una distribución normal de los microfilamentos (Saunders y Parks, 1999). Los cambios observados en la organización de los filamentos de actina podrían provocar cambios en otros componentes celulares como las mitocondrias o la membrana plasmática debido a la asociación de los microfilamentos a éstas y a otras estructuras del citoplasma (Didion *et al.*, 1990; Fuku *et al.*, 1995).

La presencia de la placa metafásica en los ovocitos maduros hace de éstos un tipo celular muy difícil de crioconservar. Esta estructura posee microtúbulos polimerizados íntimamente relacionados con los cromosomas condensados. Ambas estructuras son altamente susceptibles a la despolimerización y/o desorganización por descensos de temperatura (revisado por Aman y Parks, 1994; Bernard y Fuller, 1996). Por este motivo, diferentes autores hipotetizaron que la crioconservación de ovocitos en estadios inmaduros de desarrollo favorecería la preservación de estas estructuras ya que este tipo de ovocitos mantiene los cromosomas descondensados y protegidos por una membrana nuclear, y los microtúbulos despolimerizados y distribuidos por el citoplasma (revisado por Arav *et al.*, 1993). Sin embargo, los resultados obtenidos por Didion *et al.*(1990), Isachenko *et al.* (2001), Otoi *et al.* (1995), Parks y Ruffing (1992) y Rojas *et al.* (2004) demostraron que la crioconservación de ovocitos en este estadio de maduración daba lugar a peores resultados que la crioconservación de ovocitos maduros. Éstos resultados difieren de lo observado en nuestros estudios en terneras utilizando la vitrificación por OPS ya que tan sólo se observó una pequeña disminución no significativa en el porcentaje de división embrionaria a las 48 horas post-inseminación tras vitrificar ovocitos en estadio de GVBD comparado con aquellos vitrificados en estadio de MII. Estos resultados pueden ser explicados como una consecuencia de vitrificar ovocitos en

un estadio donde se suman las ventajas y desventajas de congelar en un estadio de maduración intermedio (Hochi *et al.*, 1998; Le Gal y Massip, 1999; Men *et al.*, 2002).

Así, en nuestro estudio, la vitrificación se llevó a cabo a las seis horas de haber iniciado la maduración, lo que permitiría al ovocito llevar a cabo la síntesis de proteínas necesarias para la fecundación y posterior desarrollo embrionario. Además, el hecho de que la GVBD no presente aún una organización estricta de los cromosomas ni de los microtúbulos nos llevó a suponer que este estadio de maduración sería menos sensible a la vitrificación. Los resultados obtenidos en nuestros experimentos demostraron que si bien los ovocitos en GVBD eran capaces de completar la primera división meiótica tras su vitrificación, el porcentaje de placas metafásicas normales no mejoró. Por tanto, podemos suponer que la vitrificación en estadio de GVBD provocó suficientes daños en los cromosomas y los microtúbulos como para que la placa metafásica resultante de la primera división meiótica presentase ya patrones de distribución de cromosomas y microtúbulos anormales.

La premaduración de los ovocitos con un inhibidor del reinicio de la meiosis permitiría que los ovocitos tuvieran una mayor oportunidad para concluir la maduración citoplasmática y así, mejorar su potencial de desarrollo embrionario (Lonergan *et al.*, 1997; Avery *et al.*, 1998; Mermillod *et al.*, 2000; Motlik *et al.*, 2000). Para tratar de mejorar el potencial de desarrollo embrionario, la literatura describe diferentes inhibidores del reinicio de la meiosis como la ciclohexamida (Lonergan *et al.*, 1997; Saeki *et al.*, 1997), la dimetilaminopurina (Lonergan *et al.*, 1997; Avery *et al.*, 1998), la butirolactona I (Kitagawa *et al.*, 1993; Motlik *et al.*, 1998; Kubelka *et al.*, 2000; Lonergan *et al.*, 2000), la roscovitina (Mermillod *et al.*, 2000), la roscovitina con ácido retinoico (Duque *et al.*, 2002) y la roscovitina con butirolactona (Ponderato *et al.*, 2001). Tras la inhibición del reinicio de la meiosis de ovocitos de ternera con roscovitina, observamos que la dosis mínima necesaria para el bloqueo meiótico era de 50 μM, con la que cerca de un 60% de los ovocitos permaneció en estadio de VG después de 24 horas de inhibición y un 74,8% de ovocitos alcanzó el estadio en MII después de 24 horas de maduración *in vitro* en ausencia del inhibidor. Este resultado fue inferior al obtenido por Mermillod *et al.* (2000) usando una concentración inferior de inhibidor (88% de los ovocitos en VG con 25 μM de roscovitina).

A nivel estructural, observamos que la premaduración de ovocitos de ternera con 50 μM de roscovitina no afectó significativamente a la estructura de la placa metafásica después de la maduración *in vitro* de los ovocitos. Sin embargo, a concentraciones superiores o inferiores (12.5, 25 y 100 μM), disminuyó significativamente el porcentaje de ovocitos que presentaban un patrón de placa metafásica normal. Estos resultados fueron similares a los obtenidos por Schoevers *et al.* (2004) quienes obtuvieron una proporción similar de ovocitos de cerdo que presentaban placas metafásicas anormales tras un proceso de premaduración con roscovitina (50μM). Por el contrario, Ju *et al.* (2003) observaron que el porcentaje de placas metafásicas anormales aumentaba tras premadurar ovocitos de cerdo con 80 μM de roscovitina. Lonergan et al. (2003) describieron cambios morfológicos en ovocitos premadurados con 125 μM de roscovitina, evidenciando la disrupción de la integridad de las células del *cúmulus*, el hinchamiento de las crestas mitocondriales y la degeneración de los gránulos corticales.

Los resultados del tercer capítulo experimental mostraron que ninguno de los dos estadios meióticos estudiados influenció la capacidad de supervivencia de los ovocitos de ternera a la crioconservación, puesto que tanto el estadio de VGBD como el de MII proporcionaron porcentajes similares de división embrionaria a las 48 horas post-inseminación. Por otro lado, la exposición de ambos estadios de maduración a los crioprotectores disminuyó el porcentaje de desarrollo embrionario obtenido. Varios autores han evidenciado que el estadio de VG es más sensible a la crioconservación comparado con los estadios maduros (Lim *et al.*, 1992; Fuku *et al.*, 1995; Zeron *et al.*, 1999; Goud *et al.*, 2000). Barnes *et al.* (1997) observaron que los ovocitos bovinos en VGBD eran más resistentes al enfriamiento que los ovocitos en estadio de GV o MII. Sin embargo, Men *et al.* (2002) obtuvieron un mayor desarrollo embrionario tras vitrificar ovocitos bovinos en estadio de MII en relación a los ovocitos vitrificados en GVBD.

A nivel estructural, pudimos observar que los ovocitos procedentes de animales prepúberes muestran un ligero descenso no significativo en el número de placas metafásicas normales después de la vitrificación y/o exposición a los crioprotectores en estadio de VGBD, comparados con aquellos en estadio de MII. El hecho de que el proceso de vitrificación de ovocitos en estadio de VGBD, por tanto con microtúbulos

descondensados, afecte a la formación de la placa metafásica después de la maduración, nos hace suponer que la crioconservación también pueda afectar a otros factores que intervienen en la formación de esta estructura (Vincent y Johnson, 1992; Wu *et al.*, 1996; Wu *et al.*, 1997).

Men *et al*. (2002) obtuvieron blastocistos tras vitrificar ovocitos de vaca en estadio de VGBD y MII mediante la técnica OPS. En este estudio, el porcentaje de blastocistos obtenido tras vitrificar ovocitos procedentes de ovarios de ternera en estadio de GVBD o MII fue nulo. Estos resultados pueden explicarse por la mayor sensibilidad que tienen los ovocitos de terneras prepúberes a la vitrificación, lo cual ya fue descrito en el primer capítulo experimental de este trabajo. Por otra parte y como ya ha sido comentado anteriormente, los ovocitos procedentes de animales prepúberes son menos competentes para el desarrollo embrionario *in vitro* que sus correspondientes de animales adultos (Revel *et al.*, 1995; Damiani *et al.*, 1996; Duby *et al.*, 1996; Khatir *et al.*, 1996; Presicce *et al.*, 1997; Taneja *et al.*, 2000).

La hipótesis de que la premaduración en bloqueo meiótico permitía a los ovocitos completar la maduración citoplasmática (Lonergan *et al.*, 2000; Mermillod *et al.*, 2000) nos llevó a pensar que al someter a los ovocitos procedentes de animales prepúberes a un proceso de premaduración con roscovitina permitiría a estos ovocitos mejorar su potencial de desarrollo embrionario y así, la vitrificación en estadio de VGBD podría ser más eficiente, debido a las ventajas estructurales anteriormente mencionadas para este estadio. Sin embargo, los resultados obtenidos demostraron que una premaduración con roscovitina no logró mejorar los porcentajes de división embrionaria a las 48 horas post-inseminación comparado con ovocitos vitrificados en estadio de MII.

La vitrificación de ovocitos de ternera tras una premaduración con 50 µM de roscovitina no incrementó el porcentaje de placas metafásicas anormales respecto a los ovocitos control pero sí disminuyó significativamente el porcentaje de división embrionaria a las 48 horas post-inseminación. Lonergan *et al* (2003) describieron daños ultraestructurales tras premadurar ovocitos bovinos con roscovitina lo que nos hace suponer que son precisamente estos daños a nivel ultraestructural causados por una premaduración con roscovitina, junto con aquellos causados por el proceso de vitrificación, los responsables de los bajos resultados en el desarrollo embrionario.

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CAPÍTULO VII:
CONCLUSIONES.

1. La vitrificación de ovocitos procedentes de ovarios de ternera y vaca provocó cambios estructurales a nivel de cromosomas, microtúbulos y microfilamentos, lo cual se tradujo en una disminución de su potencial de desarrollo *in vitro*.

2. Los ovocitos procedentes de vacas resultaron más sensibles al contacto con los crioprotectores mientras que la vitrificación afectó en mayor medida a los ovocitos de ternera.

3. Una concentración 50 μM de roscovitina permitió bloquear el reinicio de la meiosis de los ovocitos de ternera durante 24 horas sin afectar a su posterior potencial de desarrollo *in vitro*.

4. El bloqueo del reinicio de la meiosis con roscovitina (50 μM) durante 24 horas no provocó modificaciones significativas a nivel de la configuración de la placa metafásica y del citoesqueleto.

5. El estadio de maduración de los ovocitos de ternera (VGBD o MII) no tuvo influencia en los porcentajes de placas metafásicas normales, la distribución normal de los microfilamentos o el posterior desarrollo embrionario *in vitro* tras su vitrificación.

6. La vitrificación de ovocitos de ternera tras una premaduración con roscovitina no modificó los porcentajes de placas metafásicas normales ni la distribución normal de los microfilamentos, pero sí disminuyó el porcentaje de embriones *in vitro* obtenidos respecto a los ovocitos vitrificados.