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ESTRATEGIAS DE CULTIVO PARA OPTIMIZAR LA MADURACIÓN *IN VITRO* DE OVOCITOS DE TERNERAS PREPÚBERES

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INFORMA:

Que la tesis titulada "**Estrategias de cultivo para optimizar la maduración *in vitro* de ovocitos de terneras prepúberes"** presentada por Bladimir Lenin Córdova Veizaga para optar al grado de Doctor en Veterinaria, ha sido realizada bajo mi dirección y, considerándola acabada, autoriza su presentación para que sea juzgado por la comisión calificadora correspondiente.

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María Teresa Mogas Amorós

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A mis padres Roberto, Francisca
A mis hermanos Jhiovany, Carola, Alexander, Blanca
A mi Liz y nuestro tesoro

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Resumen

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El proceso de la maduración *in vitro* consiste en el mantenimiento de los ovocitos en un medio de cultivo adecuado durante un periodo de tiempo semejante al que transcurre *in vivo* desde el pico preovulatorio de LH hasta la ovulación. El éxito de la misma depende de que las condiciones que soporten los ovocitos durante este tiempo se asemejen en lo posible a las que se producen durante la maduración fisiológica en el animal. Así, en el primer trabajo, se evaluaron los efectos de la adición del complejo Insulina-Transferrina-Selenio y/o ácido ascórbico al medio de maduración *in vitro* de ovocitos de terneras prepúberes, ya que estos componentes juegan un papel muy importante frente al estrés oxidativo producido en condiciones *in vitro*. Los resultados demostraron que aquellos ovocitos que habían sido madurados en presencia del complejo Insulina-Transferrina-Selenio (ITS) y ácido ascórbico durante las 12 primeras horas, presentaban un mayor nivel de expresión de la ciclina B1, un mayor porcentaje de ovocitos que presentaban una correcta distribución periférica de los gránulos corticales y un mayor porcentaje de blastocistos.

En el segundo trabajo, se analizó la adición de leptina al medio de maduración *in vitro*. Estudios anteriores demostraron que la leptina ejercía efectos positivos durante la maduración *in vitro* de ovocitos de vacas, aumentando tanto la proporción de ovocitos que se desarrollaban a blastocistos como el número de células de estos. También se observó que el uso de leptina durante la maduración reducía tanto la proporción de apoptosis en las células del cúmulus como en las células de los blastocistos obtenidos. Así, en este estudio se analizó si la adición de leptina en distintas concentraciones incrementaba la tasa de blastocistos, porcentajes de apoptosis de las células del cúmulus y el nivel de abundancia relativa de ARN mensajero de genes involucrados con la apoptosis y la competencia del ovocito. Los resultados indicaron que la adición de la leptina al medio de maduración *in vitro* de ovocitos de terneras prepúberes no incrementaba el potencial de desarrollo embrionario, ni disminuía los niveles de apoptosis en las células del cúmulus, existiendo un incremento en los niveles de apoptosis a mayor dosis de leptina. Por otro lado, las distintas concentraciones de

leptina durante la maduración *in vitro* ocasionaron un bloqueo en la transcripción del receptor de la leptina (*LEPR*), probablemente relacionado con la degradación selectiva por las altas dosis de leptina

En el tercer trabajo se propuso la inhibición de la Catepsina B, una cisteín proteasa lisosomal que juega un papel importante en la proteólisis intracelular. Estudios recientes indicaron que la abundancia de transcripción de ARN mensajero de la catepsina B en las células del cúmulus incrementaba en los complejos cúmulus-ovocito con baja competencia de desarrollo comparado con el de los complejos cúmulus-ovocito con mayor competencia de desarrollo. Por lo tanto, en este trabajo se analizó el efecto del E-64 (trans-Epoxysuccinyl-L-leucylamido (4-guanidino) butano) inhibidor de la catepsina B, durante la maduración *in vitro* de los complejos cúmulus-ovocito de terneras prepúberes. Así, los resultados demostraron que la inhibición de la catepsina B durante la maduración *in vitro* no incrementó el potencial de desarrollo embrionario, ni la calidad del embrión. Por otra parte, se observó una tendencia a incrementar el nivel de apoptosis de las células del cúmulus a medida que aumentaba la concentración de E-64. Asimismo, la cantidad de proteína catepsina B aumentó en los ovocitos madurados en presencia de altas dosis del inhibidor y se mantuvo constante en las células del cúmulus. Finalmente, la adición de E-64 durante la maduración *in vitro* no produjo un efecto positivo en la competencia de los ovocitos de terneras prepúberes.

En conclusión, nuestros estudios indican que la adición de moléculas como la leptina o E-64 durante la maduración *in vitro* de ovocitos de ternera prepúberes no mejora su capacidad intrínseca para el desarrollo embrionario. En cambio, los resultados obtenidos indican que es posible incrementar el porcentaje de blastocistos obtenido mediante la adición del complejo Insulina-Transferrina-Selenio y ácido ascórbico al medio durante las 12 primeras horas de maduración.

Introducción

Las posibilidades de aplicación de las tecnologías reproductivas *in vitro* son numerosas y presentan un elevado interés en el caso del ganado bovino. La producción de embriones *in vitro* (PIV) a partir de ovocitos obtenidos por aspiración ovárica de vacas de elevado valor genético o en peligro de extinción es una herramienta que ofrece nuevas perspectivas para la aceleración del progreso genético o su conservación. Sin embargo, esta técnica es todavía poco eficiente, lo que dificulta su aplicación a gran escala. Actualmente, el uso de la producción *in vitro* de embriones bovinos a nivel de campo se ve obstaculizada por varios problemas, incluyendo las pobres tasas de desarrollo hasta el estadio de blastocisto, embriones de mala calidad y la reducción en las tasas de implantación en comparación con lo evidenciado en embriones producidos *in vivo* (Rizos *et al*., 2002; Lonergan *et al*., 2003). Por otro lado, la producción *in vitro* de embriones a partir de animales prepúberes ofrece ventajas como la reducción del intervalo generacional, permitiendo valorar el animal reproductor antes de llegar a la edad reproductiva y una mayor disponibilidad en el ámbito comercial. Por el contrario, está ampliamente descrito que los ovocitos de animales prepúberes presentan una menor competencia comparada con ovocitos de animales adultos (revisado por Armstrong, 2001). Esta baja eficiencia se debe, en gran medida, a las diferencias estructurales y fisiológicas que presentan los ovocitos de animales prepúberes, como la incapacidad para completar la maduración citoplasmática (Salamone *et al*., 2001), disminución del diámetro celular (Armstrong *et al*., 2001) y un menor grado de metabolismo energético (Gandolfi *et al*., 1998), enzimático y proteico (Driancourt *et al*., 1991).

La población de ovocitos que se utiliza actualmente para la producción de embriones *in vitro* a nivel de investigación es muy heterogénea ya que se obtienen de ovarios procedentes del matadero, donde se desconoce exactamente el estado fisiológico, sanitario y nutricional del animal (Wrensiky *et al*., 2007). Así, el origen y la calidad de los ovocitos es uno de los problemas más importantes dentro de la producción *in vitro* de embriones (Lonergan *et al*., 2008). Además, los embriones producidos *in vitro* son de calidad inferior comparados con los que se obtienen *in vivo* (Rizos *et al*., 2002). Existe mucha información que respalda esta afirmación basados en datos morfológicos,

criotolerancia, perfil de transcripción de ARN mensajero y por supuesto, en las tasas de gestación después de la transferencia (Lonergan *et al.*, 2006).

La influencia de la calidad del ovocito en el potencial de desarrollo del embrión ha sido reconocida en la vaca con mayor claridad que en ninguna otra especie. Se entiende como un ovocito competente a su capacidad para reanudar la meiosis, ser fecundado, desarrollarse hasta el estadio de blastocisto, inducir la gestación y el nacimiento (revisado por Sirad, 2006). En este sentido, la calidad intrínseca de un ovocito se determina por la presencia de un conjunto normal de cromosomas, una correcta reorganización de los orgánulos citoplasmáticos y el almacenamiento de ARN mensajeros para su posterior uso, la cual puede ser influida por el microambiente, ya sea tanto en el folículo preovulatorio como en condiciones de maduración *in vitro*. La maduración citoplasmática incluye una serie de cambios ultraestructurales con respecto a la morfología y la redistribución de los orgánulos citoplasmáticos, fundamentalmente de las mitocondrias (Thibault *et al.*, 1987), los gránulos corticales (Ducibella *et al.*, 1990), y los microtúbulos (Diamini *et al.*, 1996), siendo esta reorganización necesaria para el progreso de la maduración y el bloqueo de la poliespermia. El desplazamiento de orgánulos citoplasmáticos durante la maduración se produce a través de las acciones de los microfilamentos y los microtúbulos (Ferreira *et al.*, 2009). En el ovocito bovino, los cambios citoplasmáticos no se completan hasta las 30 horas de haber comenzado la maduración, aunque la maduración nuclear finaliza a las 24 horas (Hyttel *et al.*, 1988). Estos cambios citoplasmáticos incluyen, sobre todo, dos procesos: la ubicación de los gránulos corticales en la periferia, situándose bajo la membrana plasmática (Ducibella *et al.*, 1994) y el agrupamiento de las mitocondrias (Stojkovic *et al.*, 2001). En esta fase final de la maduración, sigue existiendo una alta actividad de síntesis proteica con el fin de preparar citoplasmáticamente al ovocito para la fecundación (Wrenzycki *et al.*, 2007).

Los resultados de producción *in vitro* de embriones en las distintas especies fueron mejorando significativamente a medida que avanzaron los conocimientos acerca de sus requerimientos. Para ello, fue necesario transformar los primeros medios de cultivo en medios más definidos, en los cuales cada uno de sus componentes pudiera ser estudiado en función del efecto que producía sobre la maduración del ovocito, el desarrollo embrionario, la tasa de gestación y el porcentaje de crías viables. En este sentido, y

centrándonos en la temática de la tesis, a lo largo de los años se han estudiado muchos factores que favorecen el proceso de la maduración *in vitro*, y entre ellos, los medios de maduración se han suplementado con una amplia variedad de hormonas, antioxidantes e inhibidores.

El uso de las hormonas durante la maduración *in vitro* es debido a que las gonadotropinas (FSH, LH) y algunas hormonas esteroides (estradiol 17B) tienen un papel fundamental en el desarrollo del folículo y en la maduración del ovocito (Fukui *et al*., 1983). Por otro lado, otras hormonas, como la insulina o el factor de crecimiento insulínico tipo I (IGF-I), también pueden ser añadidos a los medios de maduración *in vitro* por sus efectos positivos. La insulina estimula la síntesis de ADN y ARN, proteínas y lípidos, interviene en la regulación de funciones celulares a distintos niveles e incrementa la utilización de la glucosa (Rao *et al*., 1990). Por otro lado, se ha detectado la transcripción del receptor de la insulina en ovocitos humanos (Lighten *et al*., 1997), bovinos (Watson, 1992) y porcinos (Quesnel *et al*., 1999). Asimismo, se ha evidenciado que los embriones bovinos a partir de 8-16 células contienen receptores para la insulina (Watson, 1992), y se ha demostrado que la insulina incrementa el número de células de la masa celular interna y mejora el proceso de la blastulación en embriones de ratón (Harvey y Kaye. 1990).

La manipulación en el laboratorio y las técnicas de cultivo *in vitro* exponen a los ovocitos a condiciones ajenas a la protección antioxidante materna, como una atmosfera con abundante oxígeno, la luz o las trazas de metales pesados del medio de cultivo. Todos estos factores son responsables del incremento de las especies reactivas del oxígeno (ROS), que causa efectos nocivos en ovocitos y embriones (Guerin *et al.*, 2001). Así, para proteger a los ovocitos durante el periodo de la maduración *in vitro*, se han añadido y/o combinado varios antioxidantes para modular el estrés oxidativo extracelular, como la superoxido dismutasa o catalasa (Ali *et al*., 2003), cisteamina (de Martos *et al*., 2002), β-mercaptoethanol (de Martos y Furnus 2000; Funahashi *et al*., 2005), retinol (Duque *et al*., 2002) o ácido ascórbico (Tatemoto *et al*., 2001; Dalvit *et al*., 2005). El ácido ascórbico adicionado durante la maduración *in vitro* de ovocitos mejora el desarrollo y la calidad de los embriones mediante un descenso de la apoptosis de las células del cúmulus y una mejora de la maduración citoplasmática (Tatemoto *et al*., 2001; Wu *et al*., 2006). La transferrina, es una proteína que actúa como un

desintoxicante mediante la eliminación de metales tóxicos en los medios y actúa como un promotor del crecimiento muy importante (Bowles *et al*., 1998; Briggs *et al*., 1999). Diversos trabajos demuestran que la adición de transferrina, sola o en combinación con insulina, al medio de cultivo no tiene ningún efecto positivo sobre el desarrollo de los embriones bovinos (Siedel *et al*., 1991; Flood *et al*., 1993). Por el contrario, Bowles y colaboradores (1998) señalaron que esta proteína mejora el crecimiento celular y el porcentaje de blastocistos obtenidos después del cultivo *in vitro*, ya sea sola o en combinación con insulina y selenio. Por otro lado, se sabe que el selenio regula la actividad del glutatión peroxidasa, previniendo la generación de radicales peróxidos y por lo tanto previene el daño oxidativo a las células y tejidos (Nasr-Esfahani *et al*., 1992).

Recientemente, se ha identificado a la leptina (*LEP*) y su receptor (*LEPR*) en los ovocitos, células del cúmulus y embriones (Boelhauve *et al*., 2005; Paula-Lopes *et al*., 2007). La leptina es una hormona peptídica de 16 kDa secretada principalmente por el tejido adiposo y desempeña un papel importante en la regulación del peso corporal y el gasto energético (Fruhbeck, 2006). Además, está implicada en las funciones reproductivas de varias especies (Ryan *et al*., 2002). Estudios previos a nivel *in vitro* han indicado que la leptina juega un papel importante en el desarrollo embrionario temprano, en la regulación de expresión de genes relacionados con el metabolismo, la apoptosis y la implantación (Boelhauve *et al*., 2005). Matsuoka y colaboradores (1999) indicaron que la acción de la leptina durante la maduración de los ovocitos es a través del transductor de señal y activador de la transcripción 3 (STAT3), donde detectaron que la estimulación de ovocitos de ratón con la leptina causa fosforilación de la STAT3, lo que indica una función en la regulación de la transcripción. Por otro lado, Craig y colaboradores (2004) demostraron que la presencia de leptina en el medio de maduración *in vitro* de ovocitos porcinos incrementa la fosforilación de MAPK y de esta forma estimula la progresión de los ovocitos al estadio de metafase II. También indicaron que la leptina disminuye los niveles de AMPc en el ovocito para iniciar el proceso de la ruptura de la vesícula germinal, lo cual, conduce a la maduración nuclear del ovocito.

Por otro lado, existen estudios que indican que la inhibición de ciertas moléculas durante el proceso de la maduración *in vitro* ofrecen al ovocito inmaduro condiciones

necesarias para alcanzar una adecuada maduración nuclear y citoplasmática (Ferreira *et al*., 2009). Tal es el caso de la pre-maduración de los ovocitos con inhibidores farmacológicos de la meiosis, que tienen como función inhibir la actividad de la quinasa dependiente de ciclina (CDK), como la roscovitina (Mermillod *et al*., 2000) y butirolactona I (Adona *et al*., 2008), e inhibidores de la fosfodiesterasa que aumentan las concentraciones de AMPc, como el 3-isobutyl 1-methylxanthine (IBMX) (Aktas *et al*., 1995). Asimismo, otro inhibidor como el E-64 ha demostrado bloquear la actividad proteolítica de la catepsina B durante la maduración *in vitro* (Balboula *et al*., 2010). La actividad de la catepsina B en las células del cúmulus está relacionada con la capacidad de desarrollo del ovocito. Así, se ha observado que la abundancia de transcripción de la catepsina B en las células del cúmulus incrementa en los complejos cúmulus-ovocito de baja calidad (Bettegowda *et al*., 2008). Asimismo, estudios previos han demostrado que la maduración *in vitro* de los complejos cúmulus-ovocito en presencia de E-64 reduce el nivel de apoptosis de las células del cúmulus y aumenta la capacidad de desarrollo embrionario hasta el estadio de blastocisto (Bettegowda *et al*., 2008; Balboula *et al*., 2010).

Objetivos

El objetivo general de esta tesis consistió en analizar el efecto de la adición de distintos componentes durante la maduración *in vitro* sobre la calidad del ovocito y el potencial de desarrollo del embrión, con la finalidad de optimizar el rendimiento en la producción *in vitro* de embriones de terneras prepúberes.

Para ello, se establecieron los siguientes objetivos concretos:

Determinar los efectos de la adición del complejo Insulina-Transferrina-Selenio y/o ácido ascórbico al medio de maduración *in vitro* de ovocitos de terneras prepúberes a nivel de maduración citoplasmática y su posterior desarrollo embrionario.

Determinar si la adición de leptina en distintas concentraciones durante la maduración *in vitro* incrementa la tasa de blastocistos, porcentajes de apoptosis de las células del cúmulus y el nivel de abundancia relativa de ARN mensajero de genes involucrados con la apoptosis y la competencia del ovocito.

Determinar el efecto del inhibidor de la Catepsina B E-64 durante la maduración *in vitro* de ovocitos de terneras prepúberes sobre la capacidad y calidad de desarrollo embrionario.

Effect of the addition of Insulin-transferrin-selenium and/or L-ascorbic acid to the *in vitro* maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development

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Effect of the addition of insulin-transferrin-selenium and/or L-ascorbic acid to the *in vitro* maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development

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Abstract

This study examines the effects of adding insulin-transferrin-selenium (ITS) and/or L-ascorbic acid (ASC) to a conventional medium for maturing prepubertal calf oocytes on chromosome organization, cortical granule (CG) distribution, and embryo development to the blastocyst stage. Cumulus-oocyte complexes (COCs) were matured in medium TCM 199 containing PVA and EGF (control), and supplemented with ITS and/or ASC for 12 or 24 h at 38.5 °C in a 5% CO_2 atmosphere. Calf oocytes matured with ITS + ASC or ASC for 12 h showed significantly higher percentages of peripherally distributed CG (83.3% and 86.2% respectively) than control oocytes (71.4%) or those matured with ITS alone (71.4%). No effects on chromosome organization were detected. Conversely, 24 h of supplementation did not affect CG distribution patterns, while the addition of ASC gave rise to significantly higher percentages of oocytes showing a normal alignment of their chromosomes (72.9%) compared to controls (58.7%). At 48 hpi, similar cleavage rates were observed among treatments regardless of the treatment time. However, the presence of ITS + ASC for 12 h rendered significantly higher blastocyst rates than those recorded in the remaining groups. Supplementation for 24 h with ITS or ITS + ASC had no significant effects on the percentage of blastocysts obtained, while the presence of ASC significantly reduced the proportions of embryos developing to the blastocyst stage. Our data suggest that ITS plus L-ascorbic acid supplementation during the first 12 h of *in vitro* maturation improves cytoplasm maturation and the developmental competence of embryos produced from prepubertal calf oocytes.
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1. Introduction

In vitro maturation, fertilization, and culture procedures used for cattle follicular oocytes have been the subject of recent research and much progress has been made in this area. Combining transvaginal oocyte retrieval with the *in vitro* production of cattle embryos has been a significant leap forward with far-reaching practical implications. The use of prepubertal heifers as oocyte donors for IVP shortens the interval between generations and prolongs the reproduction period. If successful, this strategy will further benefit cattle genetic improvement programs. However, to improve the efficiency of this IVP technique, the culture systems presently used will need to be modified to suit the requirements of these juvenile oocytes.

There have been two main approaches to improve the developmental competence of oocytes after their

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recovery from the follicle. The first has been the addition of putative growth promoting substances to the culture medium (gonadotrophins, steroids, growth factors). Despite modest improvements in development achieved in this way, blastocyst yields have rarely consistently surpassed 50% [1]. The second approach has attempted to mimic the intrafollicular conditions of the oocyte through meiotic arrest. Typically, oocytes subjected to *in vitro* maturation, though capable of high rates of nuclear maturation, have had insufficient time to undergo normal cytoplasmic maturation. Hence, using a variety of cellular and chemical methods, researchers have tried to artificially inhibit the resumption of meiosis after oocytes are removed from the follicle to allow for cytoplasmic development *in vitro* in the absence of nuclear maturation (reviewed by Sirard [2]). These studies have shown that it is possible to maintain meiotic arrest outside the follicle for 24–48 h and to release the oocyte from this meiotic arrest without detrimentally affecting blastocyst or foetal development. However, none of these approaches have managed to improve developmental competence, even when embryos are produced from prepubertal animals [3].

During *in vitro* culture, cells are exposed to higher concentrations of oxygen than those that occur *in vivo* and this causes the constant production of free radicals. High levels of free radicals cause cell damage leading to loss of function [4]. Thus, to optimize embryo production, oocytes need to be protected against oxidative stress during *in vitro* culture by adding antioxidants to the culture media.

Ascorbic acid is the main water-soluble antioxidant present in the ovary, where it also serves as a cofactor for collagen synthesis and peptide amidation and facilitates follicular growth [5]. The addition of ascorbic acid to the culture medium has been shown to prevent follicular apoptosis in rat and mouse follicles, and to improve mouse blastocyst production [6,7]. Ascorbic acid also enhances the developmental competence of porcine oocytes [8] and prevents apoptosis in granulosa cells [5] and ovarian follicular cells [7]. Some authors have nevertheless reported adiaphorous effects of ascorbic acid on the *in vitro* maturation of bovine oocytes [9].

Insulin is a polypeptide hormone that promotes the uptake of glucose and amino acids and may have mitogenic effects [10]. In effect, the developmental potential of pig oocytes and embryos is enhanced when insulin and insulin like growth factors are added to the IVM and IVC media [11,12]. Selenium (Se) is an essential trace element for several physiological processes [13]. In cell culture systems, selenium in the form of sodium selenite protects the cells from oxidative damage by reducing free radical production and inhibiting lipid peroxidation [14,15]. The combination insulin–transferrin–selenium (ITS) may be used in both complex and non-complex media. For most serum-free culture media, ITS is the supplement of choice to promote the development of oocytes [16] and is routinely used in several IVM systems. Previous experiments have shown that the presence of selenium as ITS supplementation supports follicular growth and oocyte maturation *in vitro* [17,18]. In these reports, the addition of ITS to defined or semi-defined IVM media was described to improve the developmental competence of pig or buffalo oocytes. However, the effects of ITS supplementation during the IVM of prepubertal calf oocytes have not yet been determined. The present study was designed to test the hypothesis that the addition of L-ascorbic acid and the ITS complex to a chemically defined medium could improve the *in vitro* potential of prepubertal calf oocytes for nuclear and cytoplasmic maturation and embryo development.

2. Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Plastic dishes, four-well plates and tubes were obtained from Nunc (Roskilde, Denmark).

2.1. Collection of oocytes

The methods used for the *in vitro* maturation and fertilization of oocytes have been described elsewhere [19]. Briefly, ovaries from slaughtered prepubertal calves (9 mo old) were transported from a local abattoir to the laboratory in phosphate buffered saline (PBS) at 37 °C. Cumulus oocyte complexes (COCs) were obtained by aspirating 2- to 8-mm follicles. After three washes in modified PBS (PBS supplemented with 36 μg pyruvate mL^{-1}, 50 μg gentamycin mL^{-1} and 0.5 mg bovine serum albumin (BSA) mL^{-1}), groups of up to 50 COCs were placed in 500 μL of maturation medium in four-well plates and cultured for 24 h at 38.5 °C in a 5% CO_2 humidified air atmosphere. The maturation medium (MM) was TCM199 supplemented with 1 mg polyvinyl alcohol (PVA) mL^{-1}, 10 ng epidermal growth factor (EGF) mL^{-1} and 50 μg gentamicin mL^{-1}. Besides these substances, the experimental maturation media were supplemented with 5 μL/mL ITS (Invitrogen, Cat No: 41400-045) [insulin (5 μg/mL), transferrin (3 μg/mL), selenium (3 ng/mL)]

and/or 100 μg/mL L-ascorbic acid depending on the experimental design.

2.2. 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 with 25 mmol bicarbonate L^{-1}, 22 mmol sodium lactate L^{-1}, 1 mmol sodium pyruvate L^{-1} and 6 mg fatty acid-free BSA mL^{-1}). In addition, 10 μg heparin–sodium salt mL^{-1} (Calbiochem, Darmstadt, Germany) were added. Motile spermatozoa were obtained by centrifuging frozen-thawed sperm from Asturian bulls (ASEAVA, Llanera, Asturias, Spain) on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (2.5 mL 45% (v/v) Percoll over 2.5 mL 90% (v/v) Percoll) for 8 min at 700 × g at room temperature. The pellet, collected from the bottom of the 90% fraction, was washed in Hepes-buffered Tyrode's and pelleted again by centrifugation at 100 × g for 5 min. Spermatozoa were counted in a haemocytometer and diluted in an appropriate volume of fertilization medium to give a final concentration of 2×10^6 spermatozoa mL^{-1}. 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^{-1}. Plates were incubated for 22 h at 38.5 °C in a 5% CO_2 humidified air atmosphere. Variation between individual bulls was avoided by mixing equal sperm samples from the two bulls in all the experiments.

2.3. In vitro embryo culture

At approximately 22 h post-insemination (hpi), presumptive zygotes were denuded by gentle vortexing and washed four times in PBS before being transferred to 25 μL culture droplets of SOF [20] (1 embryo/μL) supplemented with FCS (10%, v/v) under mineral oil. The dishes were incubated for 8 d at 38.5 °C in a 5% CO_2, 5% O_2 humidified atmosphere. Cleavage rates were recorded at 48 hpi and blastocyst number was determined on day 8 post-insemination.

2.4. Chromosome and cortical granules immunostaining

After 24 h of *in vitro* maturation, oocytes were denuded of cumulus cells by gentle pipetting. Oocytes were treated with 0.2% (w/v) pronase to dissolve the zona pellucida, fixed in PBS containing 2% (w/v) paraformaldehyde for 30 min and washed three times in blocking solution (PBS plus 0.1% (w/v) BSA and 7.5 mg glycine mL^{-1}). Oocytes were then treated for 10 min with permeabilizing solution (25% (v/v) glycerol, 50 mm KCl mL^{-1}, 0.5 mm $MgCl_2$ mL^{-1}, 0.1 mm EDTA mL^{-1}, 1 mm EGTA mL^{-1}, 1 mm 2-mercaptoethanol mL^{-1}, 50 mm imidazole mL^{-1} at pH 6.7 and 4% (v/v) Triton X-100) and again placed in the blocking solution. Finally, the oocytes were immersed in a staining solution composed of 100 μg *Lens culinaris*-agglutinin mL^{-1} labelled with fluorescein isothiocyanate (FITC-LCA) in PBS for 30 min. Groups of ten oocytes were mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and then covered with a drop of DAPI (125 ng mL^{-1}). Slides were then sealed with nail varnish and stored refrigerated protected from the light until observation within the following two days. An epifluorescence microscope (Zeiss: Axioskop 40) was used to examine CGs and chromatin. Images were recorded on a computer.

The criteria used to define chromosome organization and CG distribution patterns have been described elsewhere [21]. Chromosome organization was classified as: (1) Normal chromosomes: condensed chromosomes clustered as a discrete bundle at the metaphase plate. (2) Dispersed chromosomes: these were scattered in the cytoplasm or dispersed in a few zones of the cytoplasm. (3) Decondensed chromosomes: chromosomes with an aberrant, less condensed appearance. (4) Absence of chromosomes: no chromosomes were observed.

Cortical granule distribution was classified as: (1) Peripheral distribution: CGs appeared close to the plasma membrane forming a monolayer. (2) Cortical distribution: most CGs were observed in the cortical area away from the plasma membrane without forming a monolayer. (3) Cortical aggregates: CGs appeared in clusters in the cortical region. (4) Absence of CGs: no CGs were observed.

2.5. SDS PAGE and Western Blotting

After 24 h of *in vitro* maturation, a total of 50 oocytes were grouped in 15 μL PBS without BSA. The samples were frozen in liquid nitrogen and stored at −80 °C until use. On the day of processing, the samples were unfrozen and homogenized in an ultrasonic bath in 10 mM Tris-HCl buffer (pH 7.4) containing 1% (w/v) SDS, 15 mM EDTA, 150 mM KF, 0.6 M sacarose, 14 mM β-mercaptoethanol, 10 μg/mL leupeptin, 1 mM benzamidine and 1 mM phenylmethyl sulfonyl fluoride (PMSF). The homogenates were then centrifuged at 10000 g during 15 min and the supernatant were recovered. Supernatants were boiled for 1 min and

Table 1
Effects of the Presence of ITS and/or L-ascorbic acid for 12 h in the *in vitro* maturation medium on the embryonic developmental potential of calf oocytes.

Treatment	Oocytes, n	Embryo development	
		Cleavage rate, n (%)	Blastocyst rate, n (%)
Control (TCM)	165	115 (69.7)	20 (12.1)a
ITS	200	131 (65.5)	25 (12.5)a
ITS+ASC	212	144 (67.9)	42 (19.8)b
ASC	167	107 (64.1)	16 (9.6)a

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

then 15 μL were transferred to the 15% SDS-polyacrylamine gel electrophoresis (SDS-PAGE). Then, the proteins were transferred to polyvinylidine difluoride membranes (PVDF). The membrane was incubated overnight with primary antibody (rabbit anti-cyclin B, H-433, polyclonal; Santa Cruz Biotechnology, Inc) at a dilution (v/v) of 1:500 at 4 °C. Subsequently, after three washes, the immunoreactivity was tested using peroxidase-conjugated goat anti-rabit secondary antibody (A120-101P; Bethyl Laboratories, Inc., Montgomery, TX, USA) for 1 h at room temperature. Proteins were detected by the enhanced chemiluminescence (ECL Plus Western Blotting Detection; GE Healthcare UK Ltd, Buckinghamshire, England) and exposed to X-ray film (Amersham Hyperfilm ECL; GE Healthcare UK Ltd, Buckinghamshire, England).

2.6. Experimental design

Oocytes were matured *in vitro* for 12 or 24 h in maturation medium (MM) supplemented with ITS, ITS plus L-ascorbic acid or L-ascorbic acid to establish 6 treatment groups. Oocytes matured *in vitro* for 24 h in MM alone served as controls. Oocytes exposed to the supplements for 12 h were left to mature for an additional 12 h in MM.

In order to evaluate embryo development, the oocytes were fertilized after 24 h of *in vitro* maturation and cultured *in vitro*. Cleavage and blastocyst rates were determined on Days 2 and 8 after fertilization, respectively. Experiments were performed as four replicates.

In order to determine cortical granule distribution and chromosome organization, oocytes from each experimental group were fixed and examined using specific fluorescent probes before observation using an epifluorescence microscope. Experiments were performed as four replicates.

In order to evaluate Cyclin B1 protein levels and considering the results obtained in the two previous experiments, only those oocytes exposed to the supplements for 12 h. were fixed after *in vitro* maturation and analysed using the western blotting technique.

2.7. Statistical analysis

Data were analyzed statistically using the Statistical Analysis Systems package (SAS, v8). Percentages of oocytes reaching the cleavage and blastocyst stages were compared by ANOVA and significant results further analyzed by the Tukey test. To compare chromosome configurations and the distributions of cortical granules, the X^2-test was used. The level of significance was set at $P < 0.05$ for all the tests.

3. Results

3.1. Effects of the supplements on the embryonic developmental potential of the oocytes

Table 1 shows the effects of 12 h of exposure to ITS and/or L-ascorbic acid in the *in vitro* maturation medium on the developmental competence of the calf oocytes. Cleavage rates for the ITS, ITS + ASC and ASC groups were similar to those recorded for the control group (65.5%; 67.9%; 64.1%, and 69.7%, respectively). However, significantly higher blastocyst rates were recorded for the ITS + ASC group (19.8%) compared to the control (12.1%), ITS (12.5%), and ASC (9.6%) groups.

Table 2 shows the effects of 24 h of exposure of the oocytes to ITS and/or L-ascorbic acid in the *in vitro* maturation medium. No significant differences in cleavage rates were observed among the control (72.6%), ITS (64.2%), ITS + ASC (74.0%), and ASC (68.3%) groups. Rates of embryos developing to the blastocyst stage were ITS (9.0%) and ITS + ASC

Table 2
Effects of the Presence of ITS and/or L-ascorbic acid for 24 h in the *in vitro* maturation medium on the embryonic developmental potential of calf oocytes.

Treatment	Oocytes, n	Embryo development	
		Cleavage rate, n (%)	Blastocyst rate, n (%)
Control (TCM)	179	130 (72.6)	29 (16.2)a
ITS	165	106 (64.2)	15 (9.0)ab
ITS+ASC	181	134 (74.0)	15 (8.3)ab
ASC	145	99 (68.3)	8 (5.5)b

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

Table 3
ITS and/or L-ascorbic acid effects during IVM on chromosome organization.

	Treatment 12 h		Chromosome alignment n (%)			Treatment 24 h		Chromosome alignment n (%)		
	n	MII, n (%)	Normal	Dispersed	Decondensed	n	MII, n (%)	Normal	Dispersed	Decondensed
TCM	39	35 (89.7)	22 (62.9)	13 (37.1)	0	47	46 (97.8)a	27 (58.7)a	19 (41.3)a	0
ITS	40	35 (87.5)	26 (74.3)	9 (25.7)	0	52	40 (76.9)b	27 (67.5)ab	12 (30.0)ab	1 (2.5)
ITS+ASC	42	36 (85.7)	26 (72.2)	10 (27.8)	0	41	35 (85.4)bc	23 (65.7)ab	10 (28.6)ab	2 (5.7)
ASC	36	29 (80.6)	19 (65.5)	10 (34.5)	0	55	48 (87.3)c	35 (72.9)b	13 (27.1)b	0

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

(8.3%), not significantly different to the control rate. In contrast, the blastocyst rate for the ASC (5.5%) group was significantly lower than this control rate (16.2%).

3.2. *Effects of the supplements on chromosome and cortical granule patterns*

Tables 3 and 4 provide details of the chromosome organization and CG distribution patterns observed in the oocytes matured *in vitro*. The data in Table 3 indicate that the presence of ITS and/or L-ascorbic acid for 12 h in the maturation medium failed to affect the percentages of oocytes reaching the metaphase II stage (MII) compared to controls. However, when in contact with ITS and/or L-ascorbic acid for 24 h, significantly lower proportions of oocytes reached MII compared to control oocytes matured in the absence of these supplements.

Following 12 h of exposure to ITS and/or ASC, in most of the oocytes the chromosomes were normally aligned and no significant differences were observed among treatments. Proportion of oocytes showing dispersed chromosomes were also similar for all the treatments tested. In contrast, when exposed for 24 h to the supplements, a normal chromosome configuration was observed in the ASC (72.9%), ITS (67.5%), and ITS + ASC (65.7%) groups, despite only the proportion recorded for the ASC group was significantly higher than in the control group (58.7%). Similarly, a significantly lower proportion of oocytes with dispersed chromosomes was observed in the ASC (27.1%) group compared to the control group (41.3%), but this proportion was similar to those detected in the ITS (30.0%) and ITS + ASC (28.6%) groups.

The migration of cortical granules to peripheral cell areas is a characteristic feature of oocytes that have achieved cytoplasmic maturation. Our data revealed significantly higher rates of oocytes showing a peripheral CG distribution in the ITS + ASC (83.3%) and ASC (86.2%) groups than the ITS (71.4%) and control (71.4%) groups after 12 h of supplementation (Table 4). However, after 24 h of exposure to the supplements, the rates failed to differ among the different groups. Percentages of oocytes showing a CG distribution pattern defined as cortical or cortical aggregates also failed to differ among the different treatments, regardless of the exposure time.

3.3. *Effects of the supplements on the expression level of cyclin B1*

The expression of cyclin B1 has been described as a marker reflecting the cytoplasmic maturation. To investigate whether the addition of ITS and/or L-ascorbic acid influenced the cytoplasmic maturation of calf oocytes, levels of cyclin B1 protein were analyzed by the Western blotting technique after 24 h of *in vitro* maturation. Figure 1 shows a specific band for cyclin B1 protein of about 62 kDa When cyclin B1 expression was compared after 12 h of exposure to the supplements, the ITS + ASC group showed a higher level of expression when compared to the TCM or ITS group

Table 4
ITS and/or L-ascorbic acid effects during IVM on cortical granule distribution.

	Treatment 12 h		CGs distribution n (%)			Treatment 24 h		CGs distribution n (%)		
	n	MII, n (%)	Peripheral	Cortical	Cortical aggregates	n	MII, n (%)	Peripheral	Cortical	Cortical aggregates
TCM	39	35 (89.7)	25 (71.4)a	8 (22.8)	2 (5.7)	47	46 (97.8)a	36 (78.3)	9 (19.5)	1 (2.2)
ITS	40	35 (87.5)	25 (71.4)a	8 (22.8)	2 (5.7)	52	40 (76.9)b	31 (77.5)	9 (22.5)	0
ITS+ASC	42	36 (85.7)	30 (83.3)b	6 (16.7)	0	41	35 (85.4)bc	28 (80.0)	6 (17.2)	1 (2.8)
ASC	36	29 (80.6)	25 (86.2)b	4 (13.8)	0	55	48 (87.3)c	38 (79.1)	9 (18.8)	1 (2.1)

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

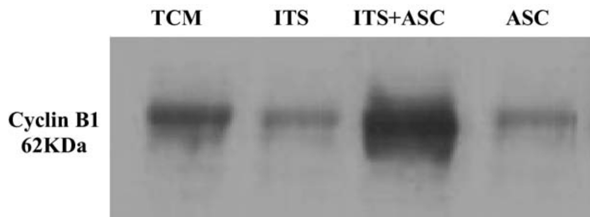

Fig. 1. Western blot analysis of cyclin B1 protein from oocytes exposed to ITS and /or ascorbic acid for 12 hours. The figure shows a representative Western blot from three independent replicates.

while the ITS or ASC groups exhibited lower intensity of signal.

4. Discussion

Juvenile *in vitro* embryo production (JIVEP) helps assess female quality before the animal reaches puberty and reduces the generational interval increasing the genetic gain rate [22]. However, several studies have revealed the reduced development competence of embryos produced using prepubertal animals as the oocyte donors [23–25]. It is known that prepubertal oocytes show a reduced developmental capacity due to their inability to complete cytoplasmic maturation and typically they do not reach the blastocyst stage since they lack sufficient amounts of RNA and proteins for further embryonic development [26]. Maturation of oocytes includes both nuclear and cytoplasmic maturation [27]. Generally, an oocyte is considered to be morphologically mature when the first polar body is extruded and the oocyte cycle is arrested at MII [28]. Cytoplasmic maturation encompasses a variety of cell processes that must be completed for oocytes to be fertilized and develop into normal embryos and offspring [29]. Damiani et al [30] reported that after *in vitro* maturation, calf oocytes show delayed migration of organelles (mainly CGs) and abnormal chromatin and microtubule configurations.

Our study sought to determine the suitability of a serum-free maturation medium supplemented with ITS and/or L-ascorbic acid for the *in vitro* maturation of calf oocytes. Oocyte maturation was assessed in terms of chromosome organization and CG distribution and the capacity of the oocytes to develop to the blastocyst stage was also assessed.

ITS added to the maturation medium had no effects on percentages of normal chromosome organization, peripheral CG distribution or embryo development, since similar results when compared to the control were observed, both for 12 and 24 h supplementation. These findings are inconsistent with those observed in pig [17] or buffalo [18] oocytes, whose developmental competence improves when ITS is added to a defined or semi-defined IVM medium. Similarly, the addition of both L-ascorbic acid and ITS to the IVM medium for 24 h did not rendered improvements in any of the factors examined. However, exposure of the calf oocytes to ITS and L-ascorbic acid in the IVM medium for 12 h, led to a significantly higher proportions of oocytes showing a peripheral CG distribution and a significantly higher percentage of *in vitro* produced blastocysts. Peripheral distribution of CGs is an indictor of cytoplasmic maturation and the consequent capacity of the oocyte to develop to the blastocyst stage [31,32]. As far as we know, the addition of both ITS and L-ascorbic acid to the *in vitro* maturation medium of prepubertal bovine oocytes has not been previously reported, though studies using porcine oocytes confirm the results obtained in our study. Thus, Wu et al [33] *in vitro* matured small follicle-derived oocytes in the presence of ITS and L-ascorbic acid for 24 h and then for an additional 20 h in their absence. These authors suggested that this maturation system improved oocyte nuclear and cytoplasmic maturation along with developmental competency compared to the conventional pig oocyte maturation system. In addition, significantly increased levels of cyclin B1 were observed in preantral oocytes matured in the presence of the ITS complex and L-ascorbic acid [33]. Oocyte maturation involves the activation of various signal transduction pathways that converge to activate the maturation promoting factor (MPF), which is composed of cyclin B and Cdc2 kinase [34]. Indeed, the amount of cyclin B present is critical for MPF activity [35]. MPF activity has been described in many mammalian oocytes: it appears just before GVBD and increases until the metaphase I stage, when its activity decreases in the anaphase–telophase stages and thereafter rises again peaking at metaphase II. The limiting factor for MPF activation has been examined in several species and species-specific differences have been identified (cattle: [36,37]). It has been suggested that the inability of oocytes to resume or complete meiosis could be related to deficient MPF activation or the lack of one of the MPF subunits. Moreover, MPF activity has been shown to be lower in prepubertal than adult female oocytes (cow: [38]; sheep: [39]). According to the results obtained from embryo development and chromosomal and CG distribution, cyclin B1 protein levels by Western blotting after 12 h of exposure to the supplements were examined. Higher levels of expression for this

protein where observed for the group ITS-ASC which suggests that a better cytoplasmic maturation can be achieved when prepubertal oocytes are matured in the presence of ITS and L-ascorbic acid for 12 h.

Aside from acting as cell antioxidants, vitamins also modulate many intracellular or extracellular biochemical processes, and are often introduced in *in vitro* oocyte and embryo culture systems to optimize results [5,6,8,40]. In the present study, the addition of ascorbic acid to the maturation medium did not modify (12 h) or even decrease (24 h) the percentage of meiotically mature oocytes regarding the control. These data are in disagreement with observations in rat and bovine oocytes, in that the presence of ascorbic acid during the whole *in vitro* maturation process did not affect nuclear maturation [9,41]. When the chromosomal configuration was analyzed, the addition of L-ascorbic acid for 24 h triggered higher percentages of oocytes with a correct alignment of chromosomes. Control group had a higher proportion of oocytes reaching the metaphase II stage, but most of these oocytes showed an abnormal chromosome alignment, whereas the group treated with L-ascorbic acid had a higher number of oocytes with normal alignment. However, the presence of higher percentages of normal chromosome configurations in the ASC group did not rendered better results and negative effects when assessing embryo development were observed. These results are opposite to the ones described by Dalvit et al [9], who observed that the supplementation of the maturation medium with ascorbic acid for 22 h did not alter the percentage of cow blastocysts produced *in vitro* regarding the control group. In porcine oocytes, Tao and co-workers observed that ascorbic acid enhanced nuclear maturation [42] and the *in vitro* developmental potential of denuded oocytes [40]. On the other hand, when oocytes were matured in the presence of L-ascorbic acid for just 12 h, periferal CGs distribution was improved but blastocyst rates where similar to the control. Differences between 12 h and 24 h of supplementation on embryo development could be attributed to cytotoxicity caused by too long exposure to L-ascorbic acid or overdose of this vitamin when juvenile oocytes are used.

In conclusion, our findings indicate that the development capacity of *in vitro* matured prepubertal oocytes was improved when oocytes were matured in the presence of the complex insulin-transferrin-selenium and L-ascorbic acid for just the first 12 h. It also revealed a positive correlation between increased expression level of cyclin B1, the distribution of cortical granules to the periphery and the higher rate of blastocysts obtained. Therefore, the addition of growth promoters and antioxidants such as insulin-transferrin-selenium and L-ascorbic acid improve cytoplasmic maturation and embryo development from prepubertal bovine oocytes.

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Effect of leptin during *in vitro* maturation of prepubertal calf oocytes: Embryonic development and relative mRNA abundance of genes involved in apoptosis and oocyte competence

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Effect of leptin during *in vitro* maturation of prepubertal calf oocytes: Embryonic development and relative mRNA abundances of genes involved in apoptosis and oocyte competence

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Abstract

During the *in vitro* maturation of adult bovine oocytes, leptin has beneficial effects on blastocyst development, apoptosis and transcription levels of developmentally important genes. The present study analyzes the differential effects of leptin on prepubertal bovine oocytes and cumulus cells. Effects were determined of leptin treatment during oocyte maturation on their developmental capacity after fertilization (Exp. 1), incidence of apoptosis in cumulus oocyte complexes (COCs) (Exp. 2) or on relative mRNA abundances of genes in cumulus cells and oocytes (Exp. 3). COCs were matured in serum-free medium containing 1 mg/mL polyvinyl alcohol and 0, 10, 100, or 1000 ng/mL leptin (L0, L10, L100, and L1000, respectively), or in medium supplemented with 10% fetal calf serum (FCS) as a positive control. Addition of leptin during oocyte maturation had no effect on cleavage rates after fertilization (FCS, 68.6%; L0, 62.9%; L10, 66.9%; L100, 63.4%; L1000, 60.9%). Similarly, no significant differences in blastocyst rates were observed when oocytes were matured in the presence of L0 (8.4%), L10 (9.3%), L100 (6.7%), L1000 (8.2%), compared to control FCS (9.4%). In Experiment 2, maturation in the presence of 1000 ng/mL of leptin increased the proportion of TUNEL-positive cumulus cell (6.9%) with respect to those matured in the presence of FCS (4.96%), but not at the lower leptin doses. When relative mRNA abundances were examined for seven genes by qRT-PCR, five (*TP53, BAX, DNMT3A, PGTS2* and *LEPR*) showed differences among groups. *LEPR* expression was significantly higher in the oocytes matured with FCS compared with the other groups and in those matured with PVA (L0) without leptin compared with the three groups of oocytes matured in the presence of leptin. In conclusion, the addition of leptin to the *in vitro* maturation medium used for prepubertal bovine oocytes does not increase the development potential of the oocytes or reduce the percentage of apoptosis in cumulus cells. Leptin blocks transcription of the leptin receptor *(LEPR)* probably reflecting selective, differential degradation by doses of leptin.
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Keywords: Bovine; Cumulus cells; TUNEL; qPCR; Prepubertal Bovine Oocytes; Leptin

1. Introduction

The *in vitro* production of embryos from young animals has been used to decrease the generation interval and thus increase the intensity of breeding selection. Prepubertal calves have also been used as a model to

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evaluate the development capacity of poor quality oocytes since they show a lower degree of oocyte competence compared to oocytes from adult animals. Reduced oocyte competence has been described in terms of lower activities of maturation-promoting factor (MPF), mitogen activated protein kinase (MAPK) and cyclin B1, alterations in protein synthesis, deficient energy metabolism, less Ca^{2+} influx at fertilization and reduced survival of embryos post-fertilization [1–5]. These biochemical and metabolic defects have thus been the subject of great concern and interest, prompting studies designed to analyze the effects of factors such as follicular fluid, serum, steroids, gonadotrophins and follicular diameter on oocyte maturation [6]. Although about 70% to 80% *in vitro* matured bovine oocytes cultured *in vitro* progress to metaphase II (MII) and complete nuclear maturation, only half of these matured oocytes reach the blastocyst stage compared to *in vivo* matured oocytes [7]. This suggests failure to achieve proper cytoplasmic maturation in *in vitro* maturation conditions. Particularly, the reorganization of cytoplasmic organelles and initiation of the synthesis of several proteins are important for the future development of the embryo [8].

In the last decade, the functional role of a leptin hormone-supplemented medium during *in vitro* maturation has been addressed in species such as mice [9], pigs [10], cattle [11–13] and horses [14]. Studies in adult cattle have shown that leptin supplementation during the *in vitro* maturation of oocytes increases the proportion of oocytes reaching the blastocyst stage and blastocyst cell numbers [11]. Although the effects of leptin on oocytes from prepubertal animals have not been investigated, Almog et al [15] found that exogenous leptin can dramatically accelerate the onset of puberty in immature female rats. These authors observed that leptin clearly attenuated follicular atresia, which may partially explain early follicular maturation, as a prerequisite for the onset of puberty.

Exposure of oocytes to physiological concentrations of leptin has been found to increase phosphorylation of the signal transducer and activator of transcription 3 (STAT3) and MAPK [10], decreasing levels of cAMP which promotes GVBD output leading to the maturation of oocytes [16]. Leptin reduces the proportion of apoptotic cumulus cells [12]. An increase in the extent of apoptosis may alter connectivity between the cells of the cumulus-oocyte subsequently reducing the quality of oocytes, and the degree of apoptosis has been correlated with the developmental competence of bovine cumulus-oocyte complexes [17]. The Bcl-2 protein family plays a key role in apoptotic control including members with opposing functions [18]. These proteins interact with each other to in some measure control apoptosis.

The leptin receptor (*LEPR*) is a membrane protein-homologous class I receptor of the cytokine family [19,20] present in oocytes [12,13], cumulus cells [12] and bovine blastocysts [11]. Binding of leptin to its receptor induces Janus kinase (JAK) associated with the receptor phosphorylation of tyrosine (Y) residues, creating phosphotyrosine docking sites for STAT proteins. After phosphorylation, tyrosine residues of these STAT proteins dissociate from the receptor to form dimers, which contribute the active transcriptional regulators. After transport into the nucleus, these regulators bind to STAT responsive elements and DNA and induce the transcription of responsive target genes [20,21].

The aims of this study were to determine the effects of leptin: 1) on the development capacity of oocytes harvested from prepubertal calves after the addition of different leptin doses (10, 100, 1000 ng/mL) to the *in vitro* maturation medium; 2) on the extent of apoptosis in cumulus cells; and 3) on relative mRNA abundances of several genes involved in apoptosis and oocyte competence.

2. Materials and methods

2.1. Chemicals and supplies

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated. Plastic dishes, four-well plates and tubes were obtained from Nunc (Roskilde, Denmark).

2.2. Collection of oocytes

The methods used for the *in vitro* maturation and fertilization of oocytes have been described elsewhere [22]. Briefly, ovaries from slaughtered prepubertal calves (9 mo of age, weight 350 to 400 Kg) were transported from a local abattoir to the laboratory in phosphate buffered saline (PBS) at 37 °C. Cumulus-oocyte complexes (COCs) were obtained by aspirating 2- to 8-mm follicles. After three washes in modified PBS (PBS supplemented with 36 μg/mL pyruvate, 50 μg/mL gentamicin and 0.5 mg/mL bovine serum albumin (BSA)), the COCs were incubated in serum-free oocyte maturation medium (TCM199 supplemented with 1 mg/mL polyvinyl alcohol (PVA), 10 ng/mL epidermal growth factor (EGF) and 50 μg/mL genta-

micin) and cultured for 24 h at 38.5 °C in a 5% CO_2 humidified air atmosphere.

2.3. *Effect of leptin on the developmental competence of prepubertal bovine oocytes*

Groups of 50 COCs were used to establish the groups: (1) FCS, oocytes matured *in vitro* for 24 h in maturation medium supplemented with fetal cow serum and epidermal growth factor; (2) L0, oocytes matured in serum-free maturation medium; (3) L10, (4) L100; and (5) L1000, COCs matured in serum-free medium with the addition of 10, 100, and 1000 ng/mL leptin, respectively (Human recombinant leptin; SIGMA ref: L4146). After *in vitro* maturation, the oocytes were fertilized and cultured *in vitro*.

2.3.1. *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 with 25 mmol/L bicarbonate, 22 mmol/L sodium lactate, 1 mmol/L sodium pyruvate and 6 mg/mL fatty acid-free BSA). In addition, 10 μg/mL heparin-sodium salt (Calbiochem, Darmstadt, Germany) were added. Motile spermatozoa were obtained by centrifuging frozen-thawed sperm from two Asturian bulls (ASEAVA, Llanera, Asturias, Spain) on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (2.5 mL 45% (v/v) Percoll over 2.5 mL 90% (v/v) Percoll) for 8 min at 700 × g at room temperature. The pellet, collected from the bottom of the 90% fraction, was washed in Hepes-buffered Tyrode's and pelleted again by centrifugation at 100 × 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 sperm/mL. A 250 μL aliquot of this suspension was then added to each fertilization well to obtain a final concentration of 1×10^6 sperm/mL. Plates were incubated for 22 h at 38.5 °C in a 5% CO_2 humidified air atmosphere. Variation between individual bulls was avoided by mixing equal sperm samples from the two bulls in all the experiments.

2.3.2. *In vitro embryo culture*

At approximately 22 h post-insemination (hpi), presumptive zygotes were denuded by gentle vortexing and washed four times in PBS before being transferred to 25 μL culture droplets of SOF [23] (1 embryo/μL) supplemented with FCS (5%, v/v) under mineral oil. The dishes were incubated for 8 days at 38.5 °C in a 5% CO_2, 5% O_2 humidified atmosphere. Cleavage rates were recorded at 48 hpi and blastocyst numbers determined on day 8 post-insemination. Each treatment was repeated nine times.

2.4. *Effects of leptin on apoptosis of cumulus cell*

To determine the effects of leptin treatment on DNA fragmentation, indicative of apoptosis in cumulus cells, the TUNEL procedure was performed using the In Situ Cell Death Detection Kit (Roche Diagnostic Corp., Indianapolis, In, USA). After IVM, COCs were washed once in 500 μL PBS containing 1 mg/mL PVP (PBS + PVP). The COCs were fixed in 4% (w/v) paraformaldehyde in PBS for 1 h and stored at 4 °C until TUNEL analysis. During TUNEL analysis, COCs were permeabilized in permeabilization solution (0.5% Triton X-100) for 2 h at room temperature. Positive and negative controls were incubated in 50 μL of RQ1 RNase-free Dnase (50 U/mL) at 37 °C for 1 h. The COCs were washed in PBS + PVP and incubated in 25 μL of TUNEL reaction mixture, which contained FITC-conjugated dUTP and terminal deoxynucleotidyl transferase for 1 h at 37 °C in the dark. Negative control COCs were incubated in the absence of terminal deoxynucleotidyl transferase. Total cell nuclei were labeled with 25 μL of Hoechst (25 μg/mL) in PBS for 30 min at 37 °C in the dark. The COCs were washed three times in PBS + PVP. Groups of five COCs were mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and then covered with a drop of mineral oil (Vectashield). Slides were then sealed with nail varnish and stored refrigerated and protected from the light until observation within the following two days. Samples were observed in an epifluorescence microscope (Zeiss: Axioskop 40) and digital images were obtained for later analysis. Total and apoptotic cells were counted in four fields per COC and data were expressed as the percentage of apoptotic cells. This experiment was repeated 6 times on 70–80 COCs per treatment group.

2.5. *Effects of leptin on specific transcript levels in oocytes and cumulus cells*

Groups of 15 denuded oocytes (in 5 μL) and their respective cumulus cells (in 15 μL) were harvested separately and stored at −80 °C until RNA extraction. The transcript levels of the genes *H2A.z, BAX, SHC1 SHC, TP53, PGTS2, DNMT3A, CCNB1*, and *LEPR* were determined by qRT-PCR analysis.

Table 1
Details of the primers used for qRT-PCR.

Gene	Primer sequence (5′-3′)	Fragment size, bp	GenBank accession no.
H2A.z	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	209	NM_174809.2
TP53	CTCAGTCCTCTGCCATACTA GGATCCAGGATAAGGTGAGC	364	NM_174201.2
BAX	CTACTTTGCCAGCAAACTGG TCCCAAAGTAGGAGAGGA	158	NM_173894.1
SHC1 SHC	GGTTCGGACAAAGGATCACC GTGAGGTCTGGGGAGAAGC	335	NM_001075305.1
DNMT3A	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	317	XM_867643.3
PTGS2	ATCTACCCGCCTCATGTTCCT GGATTAGCCTGCTTGTCTGGA	187	NM_174445.2
CCNB1	TGGGTCGCCCTCTACCCCTGC AGATGTGGCATACTTGTTCTTGATAGTCA	332	NM_001045872.1
LEPR	ACACCAGCATGATGCAGATC TCTGTAGTTGCTGGCACCAT	315	NM_001012285.2

2.5.1. *Isolation of RNA and Reverse Transcription*

Molecular biology procedures were carried out as previously described [24]. Poly(A) RNA was extracted from three groups of 15 oocytes or their corresponding cumulus cells per experimental group using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions, with minor modifications. Immediately after extraction, the reverse transcription (RT) reaction was carried out following the manufacturer's instructions (Bioline, Ecogen, Madrid, Spain) using poly(T) primer, random primers and MMLV reverse transcriptase enzyme in a total volume of 40 μL to prime the RT reaction and produce cDNA. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of reverse transcriptase. The tubes were then incubated at 42 °C for 60 min to allow the reverse transcription of RNA, followed by 70 °C for 10 min to denature the RT enzyme.

2.5.2. *Quantitative PCR (qPCR)*

The quantification of all mRNA transcripts was carried out by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). For qRT-PCR, 3 groups of cDNA per experimental group were used with two repetitions for all genes of interest. Experiments were conducted to contrast relative levels of each transcript and histone H2a.z in every sample. PCR was performed by adding a 2 μL aliquot of each sample to the PCR mix containing the specific primers to amplify histone H2Az (*H2A.z*), tumor protein p53 (*TP53*), Bcl-2-associated X protein (*BAX*), (Src homology 2 domain containing) transforming protein 1 (*SHC1 SHC*, also known as *P66*), DNA (cytosine-5) methyltransferase 3 alpha (*DNMT3A*), prostaglandin G/H synthase-2 (*PTGS2*, also known as *COX2*), cyclin B1 (*CCNB1*) and leptin receptor (*LEPR*). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. For quantification, real time PCR was performed as previously described [25]. PCR conditions were optimized to achieve efficiencies close to 1 and then the comparative cycle threshold (CT) method was used to quantify expression levels. Quantification was normalized to the endogenous control, *H2A.z*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the H2a.z CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

2.6. *Statistical Analysis*

Data were analyzed statistically using the Statistical Analysis Systems package (SAS, v8). The percentages of apoptotic cumulus cells or oocytes reaching the cleavage and blastocyst stages were analyzed by ANOVA. Significant results in the ANOVA were fur-

Table 2
Effects of the addition of leptin during *in vitro* maturation on the embryo developmental potential of calf oocytes.

Treatment	Oocytes, n	Embryo development	
		Cleavage rate, n (%)	Blastocyst rate, n (%)
FCS	468	321 (68.6)	44 (9.4)
L0	456	287 (62.9)	38 (8.4)
L10	536	359 (66.9)	50 (9.3)
L100	494	313 (63.4)	33 (6.7)
L1000	465	283 (60.9)	38 (8.2)

ther analyzed by the Tukey test. Results were considered significant at $P < 0.05$ for all tests. Relative mRNA abundance differences in oocytes and cumulus cells were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. Relative mRNA abundance differences among groups were examined by one way ANOVA.

3. Results

3.1. Effect of leptin on developmental capacity of prepubertal bovine oocytes

As shown in Table 2, there were no statistically significant differences in terms of cleavage rates between both the positive control FCS (68.6%) or L0 (62.9%) groups and the experimental L10 (66.9%), L100 (63.4%) and L1000 (60.9%) groups. Likewise, no significant differences were observed in terms of blastocyst development at Day 8 post insemination for the experimental groups L10 (9.3%), L100 (6.7%) and L1000 (8.2%) versus the FCS (9.4%) or L0 (8.4%) groups.

3.2. Effects of leptin on apoptosis of cumulus cells

Figure 1 shows the effect of leptin treatment during *in vitro* maturation on the degree of apoptosis of the cumulus cells. Oocytes matured in the presence of 1000 ng/mL of leptin showed significantly higher percentages of TUNEL positive cells (6.9%) than those matured in the presence of FCS (5.0%). Both treatments did not differ significantly from the other treatments (L0 5.8%, L10 6.1% and L100 5.6%).

3.3. Effects of leptin on levels of specific transcripts in oocytes and cumulus cells

Two genes (*PTGS2* and *LEPR*) displayed similar differences among groups in both oocytes and cumulus cells. *PTGS2* mRNA abundance was significantly higher in the group matured with FCS compared with the other groups. *LEPR* mRNA levels were significantly higher in the group matured with FCS compared with the other groups and in the group matured with PVA without leptin (L0) compared with the three groups matured in the presence of leptin. Further, the expression of the other three genes (*TP53*, *BAX* and *DNMT3A*) exhibited differences among specific groups. *TP53* mRNA abundances in oocytes were significantly higher in the group matured with FCS compared with the group matured in the presence of 10 ng/mL leptin. *BAX* expression in cumulus cells was significantly higher in the groups matured with FCS, PVA without leptin or 1000 ng/mL leptin compared with the group matured with 100 ng/mL leptin. Finally, *DNMT3A* mRNA abundances were higher in oocytes matured in the presence of FCS compared with those matured in the presence of 1000 ng/mL leptin. Similarly, in cumulus cells, *DNMT3A* was upregulated in

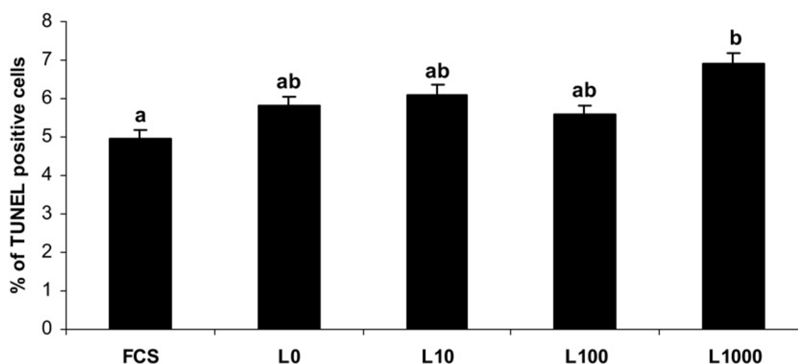

Fig. 1. Effects of leptin treatment during *in vitro* maturation on the proportion of TUNEL-positive cumulus cells. The results shown are least square means ± SEM of six replicates using 70–80 COCs per treatment. (a,b) Different letters indicate significant differences among groups ($P < 0.05$).

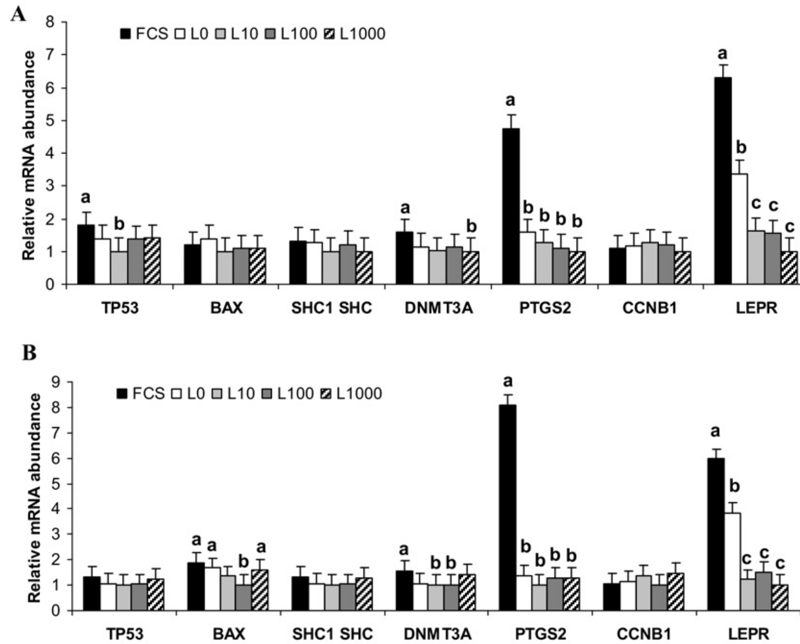

Fig. 2. Relative poly(A) mRNA abundances of 7 candidate genes related to apoptosis (*TP53*, *BAX* and *SHC1 SHC*), DNA methylation (*DNMT3A*), oocyte competence (*PTGS2* and *CCNB1*) and leptin response (*LEPR*) in cumulus-oocyte complexes matured in medium containing 10% serum (FCS) or PVA (L0) without supplementation or supplemented with three different leptin concentrations (10, 100 and 1000 ng/mL; L10, L100 and L1000 respectively). **A:** oocytes; **B:** cumulus cells. Different letters indicates significant differences between groups based on one-way analysis of variance ($P \leq 0.05$).

the group matured with FCS compared with the groups matured in 10 and 100 ng/mL leptin (Fig. 2).

4. Discussion

The present study sought to assess the effects of different concentrations of leptin added during the *in vitro* maturation of oocytes from prepubertal calves. The effects analyzed were developmental competence (cleavage and blastocyst rates), percentages of apoptotic cumulus cells, and relative mRNA abundances of genes involved in apoptosis, oocyte competence and leptin response. It has been established that the addition of leptin at physiological concentrations (~ 10 ng/mL) enhances developmental ability of *in vitro* matured adult bovine oocytes [11,12]. In a recent study, Arias-Álvarez et al. [26] reported that 10 and 100 ng/mL leptin added to the maturation medium used for adult bovine oocytes did not improve the blastocyst yield. The optimal concentration of leptin during IVM for embryo survival is still a matter of debate. Some studies suggest that leptin only may exert its effect when added *in vitro* at very high concentrations (500–1000 ng/mL) [13,27]. Thus, when different concentrations of leptin were added to the maturation medium used for adult bovine oocytes, Van Tol et al [13] observed no influence in terms of cleavage rates, while the addition of 1000 ng/mL leptin led to a significant increase in the proportion of cleaved zygotes that developed up to the 8-cell stage or blastocyst stage. In our study, different concentrations of leptin (10, 100, and 1000 ng/mL) were tested although no significant differences were observed at the level of cleavage rates or blastocyst yields. Craig et al [10] observed that pig oocytes matured in the presence of 10 ng/mL leptin gave rise to significantly higher cleavage rates after parthenogenetic activation while concentrations of 10 and 100 ng/mL significantly increased blastocyst rates. Moreover, in horses, leptin had no beneficial effect on cleavage rates after ICSI but rather, at a concentration of 100 ng/mL, it decreased the embryonic developmental rate and increased cytoplasmic fragmentation [14].

It is generally accepted that prepubertal oocytes are less developmentally competent than those from cows (reviewed by [28]). Although prepubertal calf oocytes exhibit similar rates of fertilization and cleavage to cow oocytes, their capacity to develop to the blastocyst stage is lower [29,30] and pregnancy rates from *in vivo*- or *in vitro*-produced embryos derived from oocytes of prepubertal animals are low [4,30,31]. The reduced

developmental ability has a molecular basis, as prepuberal oocytes display reduced activity of maturation promoting factors, mitogen-activated protein kinase and cyclin B, altered protein synthesis, aberrant energy metabolism, less Ca^{2+} influx at fertilization, and an overall reduced embryo survival post-fertilization [1–4,29,30,32,33]. At the transcriptional level, a microarray study reported that the transcript abundance of 416 genes differed between prepuberal and adult oocytes [34]. In this article, gene ontology revealed that it was a significant overrepresentation of transcripts encoding for genes in hormone secretion, suggesting that hormone response may differ greatly between prepuberal and adult oocytes and therefore that leptin effect may vary depending on the type of oocyte.

The incidence of apoptosis in cumulus cells may be a good indicator of oocyte developmental competence [35–37] due to the bidirectional communication established between cumulus cells and oocytes through gap junctions [38]. Cumulus cells play an important role in regulating the maturation of the nucleus and cytoplasm in oocytes [39] and in protecting oocytes against oxidative stress-induced apoptosis [40]. Several studies have revealed the occurrence of apoptosis in human [35,41] and bovine cumulus cells [17,42,43], while others have reported a lack of apoptotic cumulus cells in rats [44], pigs [45], and cattle [46]. Some authors consider that COCs with signs of early atresia are more developmentally competent [47,48] because of the similarity between structural changes during oocyte degeneration and those occurring in the oocyte of the dominant follicle prior to the LH surge [49]. Others authors, however, have reported that COCs with no signs of atresia yield higher blastocyst rates [36,37]. The present results obtained after TUNEL staining indicate that the addition of 1000 ng/mL leptin increases the level of apoptosis compared to leptin-free serum (FCS) although no significant differences were observed when compared to the rest of the groups. In adult cows, Paula-Lopes et al [12] noted that leptin enhanced the developmental potential of oocytes via cumulus cell-dependent mechanisms. These authors observed that the addition of 1 and 10 ng/mL to serum-free maturation medium reduced the proportion of apoptotic cumulus cells and assumed that leptin acted as a survival factor that rescued cumulus cells from committing to the apoptotic program [12].

The mechanisms whereby leptin suppresses the apoptotic machinery involves complex changes in the gene expression profiles of cumulus cells. To determine the effect of leptin exposure during maturation at the transcriptional level, we analyzed relative mRNA abundances of genes related to apoptosis (*TP53*, *BAX* and *SHC1 SHC*), DNA methylation (*DNMT3A*), oocyte competence (*PTGS2* and *CCNB1*) and the leptin response (*LEPR*). The mRNA abundances of two genes involved in the TP53 apoptotic pathway (*TP53* and *BAX*) varied significantly among our study groups suggesting an antiapoptotic effect of 10 ng/mL leptin for oocytes (*TP53*) and of 100 ng/mL for cumulus cells (*BAX*). Similarly, other authors have detected an antiapoptotic effect of leptin during IVM at the blastocyst stage [11], and leptin seems to prevent apoptosis in different cell types such as trophoblast cells [50], cancer cells [51] and follicular cells [9]. However, this antiapoptotic effect was not observed here by TUNEL staining which suggest that although molecular markers may detect subtle adaptations to slightly suboptimal conditions, these adaptation may not result in a significant phenotypic effect. A higher mRNA level of *de novo* DNA methyltransferase *DNMT3A* was found in both oocytes and cumulus cells compared with the different study groups matured with leptin at different concentrations. As far as we know, there are no reports of a direct effect of leptin on DNA methylation, so the transcriptional differences observed may be mediated by second order effects. Given that a higher transcriptional level of apoptotic genes was observed in the group of oocytes matured with FCS, the higher expression of *DNMT3A* may act as a compensatory antiapoptotic mechanism [52,53]. *PTGS2* was significantly upregulated in the oocytes matured with FCS compared with the rest of the groups, irrespective of the leptin concentration. mRNA abundances of this gene have been positively correlated with oocyte competence in cows [54] and humans [55,56], although here they showed no correlation with embryo development. Finally, *LEPR* mRNA abundances were lower in the groups of oocytes matured in the presence of different concentrations of leptin compared with the groups matured either with FCS or PVA alone. This downregulation of *LEPR* in the presence of leptin contrasts with the situation observed in blastocysts obtained from oocytes matured in medium supplemented with different leptin concentrations, in which a significant increase in the expression of *LEPR* in blastocysts [11] or cumulus cells [12] have been reported. Notwithstanding, the downregulation of *LEPR* in the presence of leptin has been also reported for adult tissues [57,58], which suggest that *LEPR* transcriptional regulation may depend on both the cell line and leptin concentration.

During the *in vivo* maturation of the oocytes of adult cattle, COCs are surrounded by follicular fluid leptin concentrations of around 5 ng/mL [59] while plasma concentrations range from 7 to 10 ng/mL [60]. In contrast, in prepubertal calves, blood plasma values are lower ranging from about 0.9 to 1.5 ng/mL [61]. Further, increased *LEPR* mRNA levels have been reported in both oocytes and cumulus cells obtained from cows [12,13], whereas we observed the opposite in prepubertal heifers. Both things considered, it may be suggested that contrary to the situation in adult animals, leptin plays a minor role during the *in vitro* maturation of oocytes from prepubertal animals. In conclusion, our results suggest that the addition of leptin to the *in vitro* maturation medium does not increase the embryo development potential of prepuberal bovine oocytes, nor reduce levels of apoptosis in their cumulus cells. For the higher doses of leptin in the *in vitro* maturation medium, we observed a greater extent of apoptosis and underexpression of the leptin receptor (*LEPR*) at the transcriptional level. (Figure 2).

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.theriogenology.2011.07.002.

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Impact of cathepsin B inhibition during the maturation of prepubertal calf oocytes on subsequent embryo development

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Abstract

This study examines the effects of exposing prepubertal calf oocytes to the cathepsin B inhibitor E-64 during *in vitro* maturation on embryo development and quality, apoptosis among cumulus cells and cathepsin B protein levels. Cumulus-oocyte complexes (COCs) were matured in TCM 199 medium containing foetal calf serum and epidermal growth factor (control) or in the same medium supplemented with 1 (E1), 10 (E10) or 100 (E100) μM E-64. After *in vitro* maturation, the oocytes were fertilized and cultured *in vitro*. Cleavage and blastocyst rates were determined 2 and 8 days after fertilization. No differences were observed in cleavage rates among the control (77.7%), E1 (74.3%) or E10 (72.9%) groups, while the E100 group returned significantly lower percentages (67.3%). On day 8 post-insemination (pi), the blastocyst rate was significantly lower for E100 (3.9%) compared to the other groups (12.5%, 11.5% and 8.7% for the control, E1 and E10 groups, respectively). No significant differences in embryo quality, assessed as total cell numbers, were observed among the groups (control: 135±6.3; E1: 131±8.1; E10: 137±5.3 and E100: 124±6.4). When COCs were matured in the presence of 10 or 100 μM E-64, proportions of TUNEL-positive cumulus cell (6.7% and 7.0%, respectively) were higher that observed in controls (3.5%) or in COCs matured in the presence 1 μM E-64 (3.6%). Western blotting revealed increased levels of cathepsin B in both oocytes and cumulus cells. This increase was dose-dependent for E-64 doses of 10 μM and 100 μM in oocytes but not in cumulus cells. In conclusion, our results indicate that the addition of E-64 to the *in vitro* maturation medium used for prepubertal bovine oocytes does not increase the developmental competence or quality of subsequent embryos. On the contrary, when cathepsin B was inhibited during *in vitro* maturation, both elevated levels of apoptotic cumulus cells and levels of the protein were observed.

1. Introduction

The genetic and economic benefits of the use of juvenile animals in breeding programmes have been sufficiently well-established to consider prepubertal animals as

potential oocyte donors. However, it is generally accepted that prepubertal calf oocytes are less developmentally competent than oocytes harvested from adult cows (reviewed by [1]). Thus, although prepubertal calf oocytes exhibit similar rates of fertilization and cleavage to cow oocytes, their capacity to develop to the blastocyst stage is reduced [2,3] and pregnancy rates for in vivo- or in vitro-produced embryos derived from the oocytes of prepubertal animals have been low [3-5]. This lesser developmental capacity has an underlying molecular cause, since prepubertal oocytes show the reduced activity of maturation promoting factors, mitogen-activated protein kinase and cyclin B, altered protein synthesis, aberrant energy metabolism, reduced Ca^{2+} influx at fertilization, and overall reduced embryo survival post-fertilization [2-4,6-10]. At the transcriptional level, Patel et al. [11] reported oocyte RNA transcript profiles associated with the reduced developmental competence of prepubertal calf oocytes. These same authors assessed differences in the abundance of specific RNA transcripts in the cumulus cells of bovine oocytes derived from adult versus prepubertal animals (as a model of poor oocyte quality) using microarrays. In this study, four genes of interest encoding the lysosomal cysteine proteinases cathepsins B, S, K and Z were identified as featuring greater abundances of their transcripts in the cumulus cells of oocytes harvested from prepubertal animals. Recently, cathepsins, especially cathepsin B, in cumulus cells have been negatively related to the developmental competence of bovine cumulus-oocyte complexes (COCs) [12,13]. Accordingly, the inhibition of cathepsin B during *in vitro* maturation by a highly selective cysteine proteinase inhibitor, E-64, has been found to significantly improve the developmental competence of bovine COCs and the quality of their embryos [12,13].

Cathepsin B is a lysosomal cysteine protease that plays an important role in intracellular protein degradation in lysosomes [14] whose expression has been detected in a variety of cells including liver, ovaries and cumulus cells [12,15,16]. Cathepsin B can cause apoptosis by activating caspases indirectly through the induction of mitochondrial membrane degradation, leading to translocation of mitochondria components to the cytosol [17,18]. This translocation is the main factor in the apoptotic cascade [18-20].

Cumulus cells make direct contact with the oocyte and bi-directional communication between oocytes and cumulus cells is essential for the development and functions of ovarian follicles, promoting the production of competent mature oocytes. Cumulus cells

are a subgroup of granulosa cells that surround the oocyte in an antral follicle and, because of their close proximity to the oocyte, play an important role in regulating oocyte maturation [21,22], regulating meiotic progression [23] and protecting oocytes against oxidative stress through enhancement of their ooplasmic glutathione (GSH) content [24,25]. In addition, oocyte-secreted factors have been shown to affect cumulus cell proliferation and expansion, differentiation, steroidogenesis, and gene expression in rodents [26,27]. In the cow, similar evidence points to the oocyte as an important regulator of cumulus cell phenotype and survival, with marked effects on steroidogenesis, cell proliferation and cell death [28,29]. Given the bilateral communication that occurs between the cumulus cells and oocyte, it is plausible that changes in oocyte developmental competence will affect the cumulus cell phenotype and/or gene expression.

The present study was designed to determine the effects of: 1) inhibiting cathepsin B activity during the *in vitro* maturation of prepubertal bovine oocytes on embryo development and quality; 2) cathepsin B inhibitor (E-64) on apoptosis levels in cumulus cells; 3) the addition of E-64 during *in vitro* maturation on cathepsin B protein levels in oocytes and cumulus cells.

2. Materials and Methods

2.1. Collection of oocytes

The methods used for the *in vitro* maturation (IVM) and fertilization of the oocytes have been described elsewhere [30]. Briefly, ovaries from slaughtered prepubertal calves (< 9 months) were transported from a local abattoir to the laboratory in phosphate buffered saline (PBS) at 37ºC. Cumulus-oocyte complexes (COCs) were obtained by aspirating 2- to 8-mm follicles. After three washes in modified PBS (PBS supplemented with 36 µg/mL pyruvate, 50 µg/mL gentamicin and 0.5 mg/mL bovine serum albumin (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 TCM199 supplemented with 10% (v/v) foetal calf serum (FCS), 10 ng/mL epidermal growth factor (EGF) and 50 µg/mL gentamicin.

2.2. 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 with 25 mmol/L bicarbonate, 22 mmol/L sodium lactate, 1 mmol/L sodium pyruvate and 6 mg/mL fatty acid-free BSA). In addition, 10 µg/mL heparin-sodium salt (Calbiochem, Darmstadt, Germany) were added. Motile spermatozoa were obtained by centrifuging frozen-thawed sperm from two Asturian bulls (ASEAVA, Asturias, Spain) on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (2.5 mL 45% (v/v) Percoll over 2.5 mL 90% (v/v) Percoll) for 8 min at 700 x g at room temperature. The pellet, collected from the bottom of the 90% fraction, was washed in Hepes-buffered Tyrode's and pelleted again by centrifugation at 100 x g for 5 min. Spermatozoa were counted in a haemocytometer and diluted in an appropriate volume of fertilization medium to give a final concentration of 2 x 10^6 spz/mL. A 250-µL aliquot of this suspension was then added to each fertilization well to obtain a final concentration of 1 x 10^6 spz/mL. Plates were incubated for 22 h at 38.5ºC in a 5% CO_2 humidified air atmosphere. Variation between individual bulls was avoided by mixing equal sperm samples from the two bulls in all the experiments.

2.3. In vitro embryo culture

At approximately 22 h post-insemination (hpi), presumptive zygotes were denuded by gentle vortexing and washed four times in PBS before being transferred to 25 µL culture droplets of SOF [31] (1 embryo/µL) supplemented with FCS (10%, v/v) under mineral oil. The dishes were incubated for 8 days at 38.5ºC in a 5% CO_2, 5% O_2 humidified atmosphere. Cleavage rates were recorded at 48 hpi and blastocyst numbers determined on day 8 post-insemination.

2.4. TUNEL and Hoechst assessment of cumulus-oocyte complexes matured in vitro

To determine the effects of E-64 treatment on DNA fragmentation indicative of apoptosis in cumulus cells, the TUNEL procedure was performed using the In Situ Cell

Death Detection Kit (Fluorescein; Roche Diagnostics Corporation). After IVM, COCs were washed once in 500 µL PBS containing 1 mg/mL PVP (PBS + PVP), fixed in 4% (w/v) paraformaldehyde in PBS for 1 h and stored at 4ºC until TUNEL analysis. For the TUNEL procedure, the COCs were placed in permeabilization solution (0.5% Triton X-100) for 2 h at room temperature. Positive and negative controls were incubated in 50 µL of RQ1 RNase-free Dnase (50 U/mL) at 37ºC for 1 h. The COCs were washed in PBS + PVP and incubated in 25 µL of TUNEL reaction mixture, containing FITC-conjugated dUTP and terminal deoxynucleotidyl transferase, for 1 h at 37ºC in the dark. Negative control COCs were incubated in the absence of terminal deoxynucleotidyl transferase. Cell nuclei were labelled with 25 µL of Hoechst (25 µg/mL) in PBS for 30 min at 37ºC in the dark. The COCs were washed three times in PBS + PVP. Groups of five COCs were mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and then covered with a drop mineral oil (Vectashield). Slides were then sealed with nail varnish and stored refrigerated and protected from the light until observation within the following two days. Samples were observed in an epifluorescence microscope (Zeiss: Axioskop 40) and digital images obtained for subsequent analysis. Total and apoptotic cells were counted in four fields per COC, and data were expressed as the percentage of apoptotic cells.

2.5. Blastocyst staining with Hoechst

Embryonic cells were counted in blastocysts at 8 dpi. Blastocysts were incubated with 25 µg/mL bisbenzimide staining solution (Hoechst 33342) for 30 min at 37ºC in the dark and directly transferred to a glycerol droplet on a glass microscope slide. Cell counts were performed using the digital images obtained by epifluorescence microscopy.

2.6. SDS PAGE and Western blotting

After 24 h of *in vitro* maturation, 215 oocytes and their cumulus cells were grouped separately in 15 µL PBS without BSA. The samples were frozen in liquid nitrogen and stored at -80ºC until use. On the day of processing, the cells were thawed and homogenized in an ultrasonic bath in 10 mM Tris-HCl buffer (pH 7.4) containing 1%

(w/v) SDS, 15 mM EDTA, 150 mM KF, 0.6 M sucrose, 14 mM β-mercaptoethanol, 10 μg/mL leupeptin, 1 mM benzamidine and 1 mM phenylmethyl sulphonyl fluoride (PMSF). The homogenates were then centrifuged at 10000 g for 15 minutes and the supernatants recovered. Supernatants were boiled for 1 minute and then 15 μL were transferred to gels for 10 % SDS-polyacrylamine gel electrophoresis (SDS-PAGE). This volume corresponded to 20 μg of total protein per sample. Protein concentration was determined by the Bradford method (Bradford 1976) using a commercial kit (BioRad Laboratories; Hercules, CA). After completion of SDS-PAGE, samples were transferred to polyvinylidine difluoride membranes (PVDF). Membranes were incubated overnight with a specific cathepsin B/pro-cathepsin B primary antibody (mouse monoclonal antibody to cathepsin B, Cat. No. ab58802; Abcam, UK) at a dilution (v/v) of 1:1000 at 4ºC. Subsequently, after three washes, immunoreactivity was tested using peroxidase-conjugated goat anti-rabbit secondary antibody (A120-101P; Bethyl Laboratories, Inc., Montgomery, TX, USA) for 1 hour at room temperature. Proteins were detected by enhanced chemiluminescence (ECL Plus Western Blotting Detection; GE Healthcare UK Ltd, Buckinghamshire, England) and exposure to X-ray film (Amersham Hyperfilm ECL; GE Healthcare UK Ltd.).

2.7. Experimental design

Experiment 1: Effect of E-64 on the developmental competence of COCs

Different concentrations (0, 1, 10 and 100 μM) of E-64 (E3132; Sigma) were added to the maturation medium to assess the dose-dependent effect of cathepsin B inhibitor. After 24 h of maturation, COCs were fertilized and cultured. Developmental competence was assessed by cleavage and blastocyst rates on days 2 and 8, respectively. Total cell number was also assessed on day 8 pi blastocysts. Experiments were performed as four replicates.

Experiment 2: Effect of E-64 on the apoptotic status of matured COCs

To elucidate the inhibitory effect of E-64 on cathepsin B and its relation to apoptosis, we investigated the level of apoptosis of cumulus cells after the addition of 0, 1, 10 and 100 μM E-64 during IVM. Cumulus-oocyte complexes from each experimental group

were fixed in paraformaldehyde for TUNEL analysis. The experiments were performed as four replicates.

Experiment 3: Western blot analysis of cathepsin B in IVM oocytes

To confirm the difference in amount of cathepsin B protein, Western blotting analysis was performed for oocytes and cumulus cells obtained from COCs matured in different concentrations (0, 1, 10 and 100 μM) of E-64. After 24 h of *in vitro* maturation, groups of oocytes and their cumulus cells were stored at -80°C separately and analyzed by Western blotting.

2.8. Statistical analysis

Data were analyzed using the Statistical Analysis Systems package (SAS, v8). Percentages of oocytes reaching the cleavage and blastocyst stages, total cell numbers and apoptotic cumulus cells were compared among the experimental groups by ANOVA. Factors found to differ significantly by ANOVA were further analyzed using the Tukey test. The level of significance was set at $P<0.05$ for all tests.

3. Results

3.1. Effect of E-64 on the developmental competence of COCs

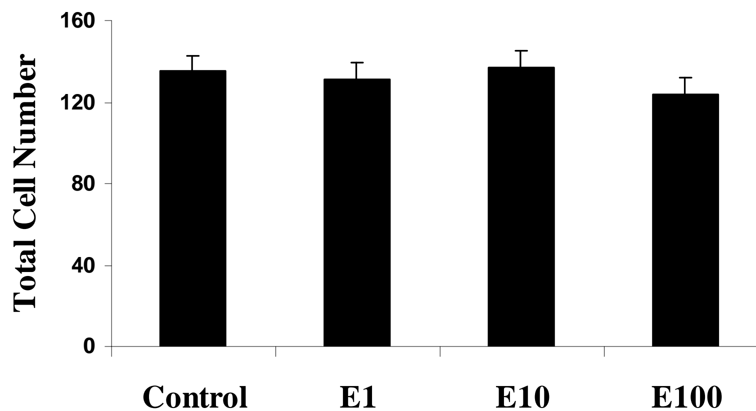
When we examined embryo development, COCs matured in the absence of the inhibitor showed a significantly higher cleavage rate (77.7%) than those matured in the presence of 100 μM E-64 (67.3%). Cleavage rates for COCs matured in the presence of 1 or 10 μM E-64 failed to differ significantly from those recorded in the other two experimental groups. In contrast, when COCs were matured in the presence of 100 μM E-64, a significantly lower blastocyst rate (3.9%) was obtained compared to those observed in the other groups (Table 1). When embryo quality was assessed in terms of total cell numbers recorded in blastocysts obtained on day 8 post-insemination, no significant differences were observed among the four groups (control: 135±6.3; E1: 131±8.1; E10: 137±5.3 and E100: 124±6.4) (Figure 1).

Table 1. Effect of cathepsin B inhibition during *in vitro* maturation on the embryo developmental potential of calf oocytes

Treatment	Oocytes, n	Embryo development	
		Cleavage rate, n (%)	**Blastocyst rate, n (%)**
Control	296	230 (77.7)a	37 (12.5)a
E1	288	214 (74.3)ab	33 (11.5)a
E10	299	218 (72.9)ab	26 (8.7)a
E100	205	138 (67.3)b	8 (3.9)b

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

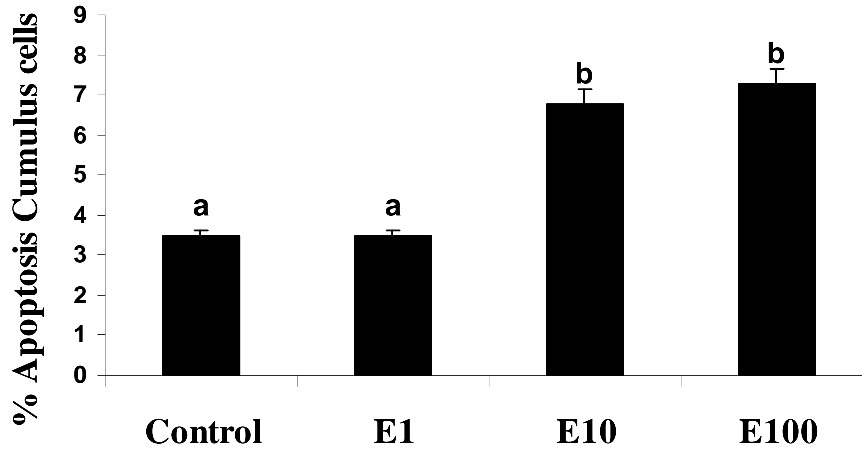
Figure 1. Effect of exposure to the cathepsin B inhibitor E-64 during *in vitro* maturation on total cell numbers in blastocysts 8 days after *in vitro* fertilization. The results shown are least square means ± SEM of four replicates using 20–30 COCs per treatment.



3.2. Effect of E-64 on the apoptotic status of matured COCs

Figure 2 shows the effect of cathepsin B inhibition during *in vitro* maturation on the level of apoptosis shown by cumulus cells. Thus, COCs matured in the presence of 10 or 100 µM of E-64 showed a significantly higher percentage of TUNEL positive cells (E10, 6.7% and E100, 7.0%) compared to the control or E1 groups (3.5% and 3.6%, respectively).

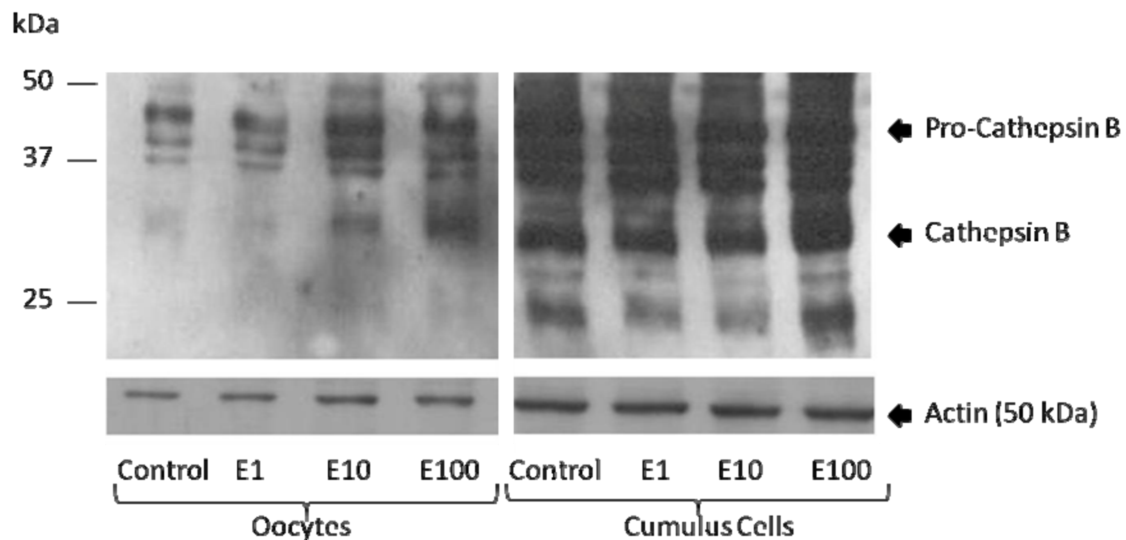
Figure 2. Effect of addition of the E-64 inhibitor during *in vitro* maturation on the proportion of TUNEL-positive cumulus cells. The results shown are least square means ± SEM of four replicates using 40–50 COCs per treatment. Values with different letters across treatments differ significantly ($P < 0.05$).



3.3. Inhibition of COC cathepsin B activity

To investigate whether cathepsin B inhibition by E-64 could affect oocyte competence, we examined cathepsin B protein levels by Western blotting 24 hours after *in vitro* maturation. Using this technique, cathepsin appeared as a band of about 30 kDa, whereas pro-cathepsin appeared as a band of about 40 kDa. Specific inhibition of cathepsin activity induced an increase in cathepsin B levels in oocytes, that was dose-dependent for E-64 doses of 10 μM and 100 μM in oocytes (Fig. 3). Interestingly, cathepsin inhibition after incubation with E-64 did not affect the amount of pro-cathepsin detected in both cell types.

Figure 3. Western blotting analysis of cathepsin B protein levels in prepubertal bovine oocytes and their cumulus cells. The figure shows a representative Western blot from three independent replicates.



4. Discussion

The lack of developmental capacity of the oocytes of juvenile ruminant species has been addressed by several authors [32-36], who attribute the developmental arrest of prepubertally derived embryos to various aspects of oocyte cytoplasm deficiency. Considering that oocytes collected from prepubertal calves, lambs or goats are usually smaller in diameter [37] than their counterparts in sexually mature females, it seems feasible that the composition of their cytoplasm will differ in morphological and/or metabolic terms from that of the mature adult female gamete. In effect, in the cytoplasm of bovine IVM oocytes and in cumulus cells, cathepsin B activity has been correlated with the quality of COCs and its inhibition was found to improve the development capacity and total cell numbers of embryos, and reduce the number of apoptotic cells [12,13]. Bearing these findings in mind, the present study was designed to assess the possibility of improving the developmental competence of embryos derived from prepubertal cow oocytes by inhibiting cathepsin B activity during *in vitro* maturation.

In our study, no effects of 1 or 10 µM E-64 were observed on cleavage rates, as reported for adult cattle oocytes matured and fertilized *in vitro* [12,13] or parthenogenetically activated [12]. However, cleavage rates fell in response to the highest concentration of

the inhibitor (100 μM). Similarly, blastocyst rates decreased with increasing concentrations of the inhibitor, although no significant differences were observed when oocytes were matured in the lower E-64 concentrations (1 and 10 μM). In contrast, other authors have reported enhanced blastocyst development following cathepsin B inhibition during the *in vitro* maturation of adult cattle oocytes [12,13] suggesting a positive effect of this strategy during IVM. As mentioned in the introduction, Bettegowda et al. [12] identified four genes of interest coding for the lysosomal cysteine proteinases cathepsins B, S, K, and Z. These genes showed greater transcript abundances in the cumulus cells surrounding oocytes harvested from prepubertal animals. The possibility that COCs from pre-pubertal animals contained a greater amount of protein than those derived from adults prompted our use of a higher inhibitor concentration (100 μM) to that used in other studies (1 μM or 10 μM). However, our results suggest a possible toxic effect of the inhibitor during the maturation period. Consistent with this idea, E-64 has been reported to prevent spindle formation during mouse meiotic maturation [38].

When we evaluated embryo quality, E-64 was found to have no effects on the total cell numbers of 8-day old blastocysts, similar to the data obtained by Bettegowda *et al*. [12]. On the contrary, however, Balboula *et al*. [13] reported that the presence of E-64 during IVM significantly increased the total cell number, and improved blastocyst quality associated with a significant increase of trophoectoderm cells.

The rate of apoptosis in cumulus cells may be a good indicator of oocyte developmental competence [1,39,40], since cumulus cells communicate with their underlying oocytes through gap junctions [2]. Cumulus cells play an important role in regulating the maturation of the nucleus and cytoplasm in the oocyte [3] and also protect oocytes against oxidative stress-induced apoptosis [41]. Cumulus cells provide the substrates used by oocytes for energy metabolism to promote oocyte meiotic maturation [27]. Through the detection of DNA fragmentation by TUNEL staining, we observed that the addition of 10 or 100 μM E-64 during the *in vitro* maturation of COCs enhanced apoptosis in cumulus cells over the levels observed for COCs matured in the absence of the inhibitor or in the presence of only 1 μM E-64. In adult cows, 1 μM or 10 μM of E-64 has been observed to improve the development potential of oocytes by impairing apoptosis in cumulus cells [12,13]. However, in the latter studies, the starting

percentage of apoptotic cells (11-16%) dropped to ~3% after treatment with 1 μM or 10 μM of E-64. In contrast, our initial rate of apoptosis was only 3.5%, such that any beneficial effect of the inhibitor would be less evident. Moreover, our higher inhibitor concentrations (10 μM or 100 μM) augmented the level of apoptosis perhaps also because of a toxic effect exerted by these concentrations on the cumulus cells.

Our Western blotting results for the detection of cathepsin B/pro-cathepsin B protein after *in vitro* maturation are not easily interpretable. In a study comparing oocytes of good versus poor quality undertaken by Balboula *et al*. [13], the authors confirmed that increased cathepsin B activity could be correlated with a greater amount of cathepsin B protein detected by Western blotting. Similar results were observed by Bettegowda *et al.* [12], who found high expression levels of mRNAs for cathepsins, including cathepsin B, in the cumulus cells of COCs from prepubertal animals. These results seem contradictory to the present data, since reduced protein levels were not detected in oocytes or cumulus cells in response to addition of the inhibitor to the *in vitro* maturation medium. On the contrary, the amount of protein increased in a dose-dependent manner in oocytes matured in the presence of the inhibitor and remained constant in cumulus cells. Although we are unable to explain these observations, we could postulate that the elevated total cathepsin B levels recorded in the oocytes are the consequence of a greater extent of cathepsin B activity inhibition, as these cells attempt to recover physiological levels of cathepsin B. Interestingly, the total amount of pro-cathepsin B in oocytes seemed not be affected by cathepsin activity inhibition. This could be the outcome of two possible mechanisms. Thus, the increase in total cathepsin B levels could be achieved through a slower degradation rate of this protein, without modifying the rhythm of maturation from pro-cathepsin B to cathepsin B. According to the second proposed mechanism, the increase in total cathepsin B could be the consequence of a synchronized increase in the rates of both pro-cathepsin B synthesis and pro-cathepsin B transformation to cathepsin B. Further experiments in which mRNA levels for pro-cathepsin are determined are needed to address this point.

In conclusion, our findings indicate that exposure of prepubertal bovine oocytes to E-64 during *in vitro* maturation is not a useful strategy to improve embryo developmental competence or embryo quality. In the presence of the inhibitor, levels of apoptosis in cumulus cells and cathepsin B protein levels were augmented. Significant differences

between cumulus-oocyte complexes derived from cows or calves in terms of size, energy metabolism, protein synthesis and the relative abundance of genes during *in vitro* maturation [4,11,42] are thought to be responsible for the variability observed in the response to various compounds (antioxidants, hormones, inhibitors, etc.) added during *in vitro* maturation to improve the efficiency of assisted reproductive technology. Hence, future studies are needed to determine the main functions of cathepsin B in cumulus-oocyte complexes harvested from prepubertal animal species as a method of improving the selectivity or specificity of its inhibitors.

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Discusión General

El potencial de desarrollo de los embriones producidos *in vitro* está limitado por defectos en las cualidades intrínsecas de los ovocitos y por las condiciones del medio de maduración. En este sentido, el objetivo general de esta tesis consistió en buscar estrategias de cultivo para optimizar la maduración *in vitro* de ovocitos de terneras prepúberes, para lo cual se analizó el efecto de distintos componentes añadidos durante la maduración *in vitro* sobre el potencial de desarrollo embrionario.

Durante maduración *in vitro* existen procesos oxidativos de los componentes celulares, que conllevan al incremento de las especies reactivas del oxigeno en el microambiente, causando alteraciones a nivel nuclear y citoplasmático. Así, en el primer trabajo, se determinó estudiar los efectos de la adición del complejo Insulina-Transferrina-Selenio y/o ácido ascórbico al medio de maduración *in vitro* de ovocitos de terneras prepúberes. La suplementación de los medios de maduración *in vitro con* estos componentes mejoró la maduración citoplasmática observándose un incremento del porcentaje de ovocitos con un patrón de distribución periférica de los gránulos corticales. Asimismo, incrementó la síntesis y almacenamiento de la ciclina B1 que contribuye a una mayor actividad del MPF. El MPF juega un papel muy importante en la progresión de la maduración meiótica del ovocito y es el responsable de la desestructuración de la lámina nuclear y de los nucléolos, de la condensación cromosómica y la reorganización de los microfilamentos (Gandolfi *et al*., 1998). Sin embargo, se ha demostrado que los ovocitos de terneras prepúberes presentan menor actividad del MPF lo cual se traduce en un descenso de la competencia del ovocito (Salamone *et al*., 2001). En nuestros resultados existe una relación positiva entre el incremento del nivel de expresión de la ciclina B1, el porcentaje de ovocitos que presentan una distribución periférica de los gránulos corticales tras la maduración y la mayor tasa de blastocistos obtenidos, sugiriendo un efecto beneficioso sobre la maduración citoplasmática y la competencia de los ovocitos. Es necesario aclarar que esta relación positiva que observamos fue en aquellos ovocitos madurados con el complejo ITS y ácido ascórbico durante las primeras 12 horas y las restantes 12 horas de maduración se llevaron a cabo en el medio control, mientras que aquellos ovocitos que fueron madurados durante 24 horas con ITS y ácido ascórbico presentaron una disminución en las tasas de blastocistos. Esta

diferencia entre periodos de exposición (12 y 24 h) puede atribuirse a que el ácido ascórbico es inestable en varias condiciones oxidativas como la exposición a pH neutro, calor, luz, y metales pesados. Una sobreexposición a ácido ascórbico termina en su degradación rápida y puede ser nocivo para los ovocitos de terneras prepúberes. Por otro lado, la suplementación solamente con ITS de los medios de maduración *in vitro* ha demostrado tener efectos positivos para la competencia de los ovocitos en las distintas especies (Raghu *et al*., 2002; Jeong *et al*., 2008). Pero en nuestro estudio, la maduración *in vitro* de ovocitos de terneras prepúberes suplementados con ITS durante 12 o 24 horas no demostró una mejora a la hora de evaluar el desarrollo embrionario hasta el estadio de blastocisto. Asimismo, la suplementación de ácido ascórbico durante las primeras 12 horas o 24 horas de maduración *in vitro* no mejoró la competencia de los ovocitos.

El efecto de la combinación ITS y ácido ascórbico no sólo depende de la concentración, sino también del tiempo de exposición con los ovocitos durante la maduración *in vitro*. Se ha indicado que la insulina estimula la síntesis de ADN, ARN, proteínas y lípidos, ayudando la maduración de los ovocitos y que la sobreexposición en los medios de maduración *in vitro* podría disminuir su potencial. Por otro lado, hemos mencionado anteriormente que el ácido ascórbico se degrada muy fácilmente en condiciones de cultivo *in vitro*. Por lo tanto, el incremento de las tasa de blastocistos en los grupos de ovocitos madurados con ITS y ácido ascórbico durante 12 horas muestran una mayor eficacia y optimización de los efectos antioxidantes del ácido ascórbico, selenio y transferrina y de los efectos mitogenéticos de la insulina.

El efecto de la adición de la leptina durante la maduración *in vitro* es muy contradictorio. Se ha indicado que la leptina promueve la maduración en los ovocitos de vacas (Paula-Lopes *et al*., 2007) y cerdos (Craig *et al*., 2004) y además, mejora el desarrollo embrionario hasta el estadio de blastocisto (Boelhauve *et al*., 2005), pero no en ratones (Swain *et al*., 2004) y ratas (Duggal *et al*., 2002). Nuestros resultados demostraron que la adición de la leptina al medio de maduración *in vitro* no incrementó el potencial de desarrollo embrionario de ovocitos de terneras prepúberes, ni disminuyó los niveles de apoptosis en las células del cúmulus, existiendo un incremento en los niveles de apoptosis a mayor dosis de leptina. Estos resultados difieren de los observados por Paula-Lopes y colaboradores (2007) donde indican que la adición de 1-

10 ng/mL de leptina en los medios de maduración *in vitro* reduce la proporción de células del cúmulus apoptóticas. Por otro lado, las distintas concentraciones de leptina durante la maduración *in vitro* ejercieron un bloqueo en la transcripción del receptor de la leptina (*LEPR*), probablemente relacionado con la degradación selectiva por las altas dosis de leptina. Estos resultados difieren de estudios previos donde demostraron que la leptina incrementaba la expresión del LEPR en células del cúmulus durante la maduración *in vitro,* indicando un efecto positivo hacia el ovocito a través de mecanismos dependientes de las células del cúmulus, posiblemente para prevenir la aparición de la apoptosis en las células del cúmulus (Paula-Lopes *et al*., 2007; van Tol *et al*., 2008).

Corroborando resultados de trabajos previos en ovocitos de bovinos adultos (Boelhauve *et al*., 2005; van Tol *et al*., 2008) y de otras especies (Craig *et al*., 2004; Lange Consiglio *et al*., 2009) hemos detectado la expresión ARN mensajero del receptor de la leptina (*LEPR*) en ovocitos y células del cúmulus de terneras prepúberes en todos los grupos experimentales. Esto indica que, independientemente de la concentración de la leptina, todos los ovocitos y células del cúmulus sintetizan ARNm de LEPR y pueden ser sensibles a esta hormona. Por otro lado, es muy importante aclarar que gran mayoría de la actividad o efecto de la leptina está en función a la unión con su receptor en la membrana y que una reducción en la transcripción del receptor de la leptina puede implicar un menor efecto sobre la capacidad de desarrollo de los ovocitos. En este sentido, Arias-Álvarez y colaboradores (2010) indicaron que los cambios en la expresión de la abundancia relativa de ARN mensajero de LEPR solo ocurre cuando existe un incremento o una disminución del rango de concentración de la leptina (0-10 ng/mL) durante el periodo de maduración *in vitro*. Además, estos cambios de expresión en los receptores de la leptina en los ovocitos durante la maduración, implican el almacenamiento de las transcripciones para actuar durante el desarrollo embrionario (van Tol *et al*., 2008). En nuestros resultados la abundancia de ARNm de LEPR disminuyó en los grupos de ovocitos madurados en presencia de diferentes concentraciones de leptina (10, 100, 1000 ng/mL). Esta baja regulación de LEPR en presencia de leptina contrasta con la situación observada por Boelhauve y colaboradores (2005) en blastocistos obtenidos a partir de ovocitos madurados en un medio suplementado con 1-10 ng/mL de leptina. Pero, estos mismos autores observaron un descenso en la transcripción de LEPR cuando se maduraron con 100 ng/mL de leptina.

No obstante, la disminución de la transcripción de LEPR en presencia de leptina se ha reportado en otros tejidos como el hipotálamo (Martin *et al*., 2000) y glándula suprarrenal (Tena-Sempere *et al*., 2000), que sugieren que la regulación transcripcional LEPR puede depender tanto de la línea celular y de la concentración de leptina.

En nuestro tercer trabajo adicionamos a los medios de maduración *in vitro* diferentes concentraciones de E-64, un inhibidor de la catepsina B. En los resultados obtenidos se observó una tendencia a incrementar el nivel de apoptosis de las células del cúmulus y un descenso en las tasas de blastocistos a medida que aumentaba la concentración de E-64. Estos resultados difieren de los descritos en trabajos previos con ovocitos de vacas adultas (Bettegowda *et al*., 2008; Balboula *et al*., 2010), donde se observó un incremento del porcentaje de embriones obtenidos así como una mejora en la calidad de blastocistos medida como un incremento en el número total de células (Balboula *et al*., 2010). Por otro lado, cuando cuantificamos la proteína presente en los ovocitos madurados en presencia del inhibidor E-64 mediante la técnica Western-blot, la cantidad de la proteína catepsina B incrementó en los ovocitos a medida que aumentaba la dosis del inhibidor mientras que se mantuvo constante en las células del cúmulus. Asimismo, se mantuvieron constantes los niveles de la pro-catepsina en los ovocitos y células del cúmulus. Se ha indicado que las catepsinas son sintetizadas en forma de pre-proenzimas. Después de la síntesis, el prepéptido se elimina durante el paso al retículo endoplasmático y las pro-catepsinas se someten a procesos proteolíticos para llegar a la forma activa de la enzima madura en el ambiente ácido de los lisosomas. Estudios previos realizados por Balboula y colaboradores (2010) detectaron diferencias en la cuantificación de la proteína catepsina B en ovocitos de buena y mala calidad tras su maduración sin la presencia del inhibidor E-64. Además, estos mismos autores relacionan el aumento de la proteína catepsina B con un incremento de la actividad proteolítica de la catepsina B en ovocitos de bovinos adultos de baja calidad y que la inhibición ejercida por E-64 durante la maduración *in vitro*, reduce la actividad proteolítica de la catepsina B e incrementa el desarrollo embrionario hasta el estadio de blastocisto. Sin embargo, en nuestros resultados se indicó que el aumento total de la proteína catepsina B en los ovocitos, puede ser una consecuencia de la disminución de la actividad de la catepsina B, inducida por la incubación con el inhibidor E-64. Podemos especular que esta inhibición de la actividad altera el ciclo biológico de la catepsina B, reduciendo el proceso de degradación natural, ocasionando la acumulación

de esta proteína en el ovocito. Por lo tanto, si bien se logra inhibir la actividad de la catepsina B, no se logra incrementar las tasas de desarrollo embrionario.

Conclusiones Generales

Las conclusiones de esta tesis son las siguientes:

1.- La capacidad de desarrollo *in vitro* de los ovocitos de ternera prepúber mejoró cuando los ovocitos fueron madurados en presencia del complejo Insulina-Transferrina-Selenio y ácido ascórbico durante las primeras 12 h. Asimismo, se observó una correlación positiva entre un incremento del nivel de expresión de ciclina B1, la distribución de los gránulos corticales en la periferia y una mayor tasa de blastocistos.

2.- La suplementación durante 24 h con ITS o ITS+ASC no tuvo efectos significativos en el porcentaje de blastocistos obtenidos, mientras que la presencia de ASC redujo significativamente la proporción de los embriones que se desarrollaron hasta la etapa de blastocisto.

3.- La incorporación de la leptina en el medio de maduración *in vitro* utilizado para los ovocitos de terneras prepúberes no aumentó su potencial de desarrollo embrionario ni redujo el porcentaje de apoptosis en las células del cúmulus.

4.- La leptina añadida al medio de maduración bloqueó la transcripción de los receptores de leptina (*LEPR*), probablemente reflejando una degradación selectiva de las dosis de leptina utilizadas.

5.- La incorporación de E-64 al medio utilizado para la maduración *in vitro* de ovocitos de terneras prepúberes no aumentó la competencia de desarrollo o la calidad de los embriones obtenidos. Por el contrario, la inhibición de la catepsina B durante la maduración *in vitro* evidenció un aumento de los niveles de apoptosis de las células del cúmulus.

6.- La maduración de los ovocitos de ternera prepúber en presencia del inhibidor E-64 provocó un aumento de los niveles de la proteína catepsina B que fue dosis-depentiente en los ovocitos para las concentraciones de 10 µM y 100 µM.

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