Tesis doctoral

# Estudio molecular y de apoptosis en ovocitos de cabras prepúberes y su relación con el desarrollo embrionario

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CERTIFICAN

Que el trabajo de investigación titulado “Estudio molecular y de apoptosis en ovocitos de cabras prepúberes y su relación con el desarrollo embrionario”, realizado por Begoña Anguita Bustamante, se ha llevado a cabo bajo su dirección en la Unidad de Producción Animal del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona para optar al grado de doctor, y gracias a la financiación del Ministerio de Ciencia y Tecnología (proyectos AGL-2000-0353 y AGL-2004304737-C03-01-GAN) y a la beca predoctoral otorgada por la Generalitat de Catalunya (2003FI 00282).

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## RESUMEN

En nuestro laboratorio siempre se ha trabajado con la finalidad de conseguir el máximo número de blastocistos posibles a partir de ovocitos recuperados de cabras prepúberes sacrificadas en matadero. La población ovocitaria recuperada de estas hembras suele ser muy variable, debido básicamente a factores fisiológicos. Por este motivo, los ovocitos deben seleccionarse cuidadosamente con el fin de utilizar en el procedimiento de producción *in vitro* de embriones sólo aquéllos capaces de madurar *in vitro*, se fecundados y mantener el desarrollo embrionario. Hasta el momento, la selección realizada en nuestro laboratorio se basaba en criterios morfológicos, seleccionando sólo los ovocitos de gran tamaño, con citoplasma homogéneo y rodeados de varias capas compactas de células del cumulus. Sin embargo, el porcentaje de blastocistos obtenidos a partir de los ovocitos seleccionados siguiendo estos criterios ha sido bajo, y no se ha conseguido superar el 10% (Izquierdo et al., 1999). Esta tesis, por lo tanto, nació de la necesidad de encontrar marcadores que pudieran indicarnos la competencia del ovocito, de modo que podamos distinguir los ovocitos competentes para el desarrollo del resto de ovocitos que no son capaces de desarrollarse hasta el estadío de blastocisto.

Los dos primeros trabajos de esta tesis, que podemos englobar dentro de un gran bloque, tiene como objetivo estudiar el papel que juegan la expresión de las subunidades del MPF (Maturation Promoting Factor), la Ciclina B1 y la $p34^{cdc2}$, así como su actividad kinasa, y la acumulación de RNA y proteínas en los ovocitos con la adquisición de competencia para el desarrollo. Las técnicas utilizadas para evaluar estos parámetros son técnicas invasivas, de modo que los ovocitos analizados no pueden ser utilizados posteriormente para continuar el desarrollo embrionario. Por lo tanto, era necesario relacionar las características moleculares estudiadas con un parámetro que nos permitiera seleccionar los ovocitos de forma visual. En estos trabajos el parámetro no invasivo escogido fue el diámetro ovocitario, ya que en numerosas especies se ha observado que existe una relación

positiva entre la capacidad del ovocito para dar lugar a un embrión viable y el diámetro folicular (bovino: Furher et al., 1989; caprino: Crozet et al., 1995; porcino: Marchal et al., 2002), y éste, a su vez, se correlaciona con el diámetro ovocitario (bovino: Arlotto et al., 1996; caprino: Crozet et al., 2000; de Smedt et al., 1994). De este modo, los ovocitos de cabras prepúberes recuperados de matadero fueron divididos en 4 grupos según su tamaño: <110 µm, 110-125 µm, 125-135 µm y >135 µm (de Smedt et al., 1994). Estos ovocitos fueron madurados y fecundados *in vitro*, y los embriones resultantes fueron cultivados *in vitro* para comprobar la competencia ovocitaria de cada grupo de tamaño. A su vez, se evaluó el estadío nuclear de una muestra de ovocitos de cada grupo antes y después de la MIV. El estudio de expresión de las subunidades del MPF se realizó mediante RT-PCR y Western blot para la $p34^{cdc2}$. Además, se cuantificó la cantidad de proteínas y de RNA acumulados en ovocitos de cada grupo mediante el método de Lowry modificado y por espectrofotometría, respectivamente. Los resultados mostraron que en el momento de recoger los ovocitos de los folículos, la mayoría ya había reanudado la meiosis, y esta proporción era mayor a medida que aumentaba el diámetro ovocitario. Además, los ovocitos de mayor tamaño fueron los que alcanzaron mayor porcentaje de maduración nuclear tras la MIV, tuvieron mayor tasa de fecundación normal, de división embrionaria a las 48 h post-fecundación, y dieron lugar al mayor porcentaje de blastocistos. Nuestros resultados mostraron que los ovocitos de mayor diámetro, que eran los más competentes, no diferían del resto de grupos estudiados en cuanto a la expresión de ARN y ARNm de $p34^{cdc2}$, de ARNm de Ciclina B1 y de proteína total, antes y después de la MIV; por el contrario, sí observamos mayor niveles de ARN total acumulado y de ARN de Ciclina B1 después de la MIV, mayor nivel de proteína $p34^{cdc2}$ y de actividad del complejo MPF. Por lo tanto, nuestros resultados indicaron que la competencia para el desarrollo estaba relacionada con el MPF, sugiriendo un posible papel de este complejo en la maduración citoplasmática.

El tercer trabajo surgió a raíz de los resultados obtenidos en los primeros estudios. Nos sorprendió observar que la mayoría de ovocitos

hubieran reanudado la meiosis en el momento de recuperarlos de los folículos. Los ovocitos sólo reanudan la meiosis en el interior de los folículos como respuesta al estímulo hormonal de la LH, previo a la ovulación, o bien debido a un proceso de degeneración o atresia. Nuestros estudios se realizan en hembras prepúberes, todavía no maduras hormonalmente, de modo que nos planteamos la posibilidad de que los ovocitos que estábamos recogiendo estuviesen sufriendo un proceso más o menos avanzado de atresia, que posiblemente pudiera interferir en los resultados de desarrollo embrionario. La realización del tercer estudio se realizó en la Universidad de Gante, donde utilizaban técnicas de detección de apoptosis de modo rutinario, y se usaron ovocitos bovinos debido a la imposibilidad de disponer de ovocitos caprinos. La finalidad de este trabajo fue aprender las técnicas de tinción con Annexin V y TUNEL mediante la realización de un estudio de apoptosis en ovocitos bovinos, células del cumulus y blastocistos según el diámetro ovocitario. Para ello, los ovocitos recuperados de matadero se clasificaron en tres grupos de tamaño: <110 µm, 110-120 µm y >120 µm (Fair et al., 1995). Los ovocitos fueron madurados y fecundados *in vitro*, y los embriones fueron cultivados durante 6 días más. Una muestra de ovocitos y células del cumulus fueron usados para evaluar apoptosis antes y después de la MIV mediante Annexin V y TUNEL. El resto de los ovocitos fueron fecundados *in vitro* y continuaron el proceso de desarrollo embrionario, y los blastocistos resultantes también fueron evaluados con las mismas técnicas. En este estudio la capacidad para el desarrollo embrionario se conseguía en los ovocitos >110 µm. Los resultados obtenidos indicaron que el porcentaje de ovocitos inmaduros que mostraban signos de apoptosis disminuía en los ovocitos >110 µm. Además, el porcentaje de ovocitos apoptóticos se redujo claramente durante la maduración *in vitro*. Por el contrario, la apoptosis en las células del cumulus aumentó con la maduración. Por último, los blastocistos obtenidos mostraron menor incidencia de apoptosis en estadío tardío en los ovocitos de mayor diámetro evaluado mediante Annexin V, pero no mediante TUNEL. A partir de estos resultados llegamos a la conclusión que las diferencias de desarrollo embrionario observadas entre ovocitos de diferente tamaño no podían ser

explicados únicamente por una diferencia en la incidencia de apoptosis entre diámetros.

Por último, el cuarto y quinto trabajo pretendían evaluar la apoptosis en ovocitos caprinos de diferente diámetro mediante las dos técnicas utilizadas en el estudio anterior. En estos trabajos quisimos introducir un parámetro más a tener en cuenta, además del diámetro ovocitario, y por ello clasificamos nuestros ovocitos también según criterios morfológicos en dos grupos: los que no mostraban signos de atresia, es decir, que presentaban el cumulus compacto y el citoplasma homogéneo; y aquellos que presentaban signos de atresia temprana, es decir, con el cumulus ligeramente expandido y/o el citoplasma del ovocito heterogéneo. Pensamos que era interesante introducir el parámetro de la morfología porque podría ser utilizado como criterio predictivo de la calidad de los embriones resultantes. Para ello, los ovocitos fueron clasificados por diámetro siguiendo los criterios explicados en el primer trabajo, y por morfología según los parámetros anteriormente mencionados. Al igual que en el anterior estudio, la apoptosis fue evaluada en ovocitos y células del cumulus antes y después de la maduración, y en los blastocistos obtenidos. En estos estudios, al igual que el realizado en bovino, también observamos una disminución de la apoptosis en los ovocitos y un incremento en las células del cumulus durante la maduración *in vitro*. En general, se observó que los ovocitos de mayor diámetro y de morfología sana presentaban menos incidencia de apoptosis, aunque los resultados obtenidos difirieron según la técnica utilizada. No se detectaron diferencias en cuanto a incidencia de apoptosis en los blastocistos obtenidos a partir de ovocitos de diferente diámetro. Por último, pudimos observar que la capacidad para el desarrollo embrionario no dependía sólo del diámetro del ovocito, sino también de su morfología. Por lo tanto, pudimos concluir que la apoptosis sí puede influenciar la capacidad para el desarrollo del ovocito, pero no la calidad del blastocisto obtenido.

## SUMMARY

In our laboratory, we have always worked in order to achieve as many blastocysts as possible from oocytes obtained from slaughtered prepubertal goats. Oocyte population recovered from these females use to be very variable, basically due to physiological factors. For this reason, we must select oocytes very carefully in order to use only the oocytes capable to mature *in vitro*, be fertilized and maintain embryonic development. So far, selection performed in our laboratory was based on morphological criteria, and we only selected large oocytes that were surrounded by several layers of compact cumulus cells and had homogeneous cytoplasm. However, the blastocyst rate obtained using the oocytes selected with that criteria has been low, and we have not achieved more than 10% (Izquierdo et al., 1999). This thesis, as a consequence, was born because we needed to find some markers of oocyte competence, so we could distinguish developmental competent oocytes from the rest of oocytes incapable to develop until blastocyst stage.

The aim of the first two works of this thesis, which can be grouped into one chapter, was to study the role that plays the expression of the subunits of MPF (Maturation Promoting Factor), Cyclin B1 and $p34^{cdc2}$, MPF kinase activity and the storage of RNA and proteins in oocytes in the acquisition of developmental competence. The methods used to evaluate these parameters were invasive ones, and therefore the oocytes evaluated could not longer be used to continue the subsequent embryonic development. As a consequence, it was necessary establish a relationship between the molecular characteristics studied with a parameter that allowed us to select the oocytes visually. Oocyte diameter was chosen as non-invasive parameter, because a relationship between oocyte competence to develop into a viable embryo and follicular diameter has been observed in many species (bovine: Furher et al., 1989; caprine: Crozet et al., 1995; porcine: Marchal et al., 2002) and the follicular diameter has been also related to oocyte diameter (bovine: Arlotto et al., 1996; caprine: Crozet et al., 2000; de Smedt et al., 1994). That way, prepubertal goat oocytes recovered from the slaughterhouse were divided in

four groups depending on their diameter: <110 µm, 110-125 µm, 125-135 µm and >135 µm (de Smedt et al., 1994). These oocytes were matured and fertilized *in vitro*, and the resulting embryos were cultured to evaluate the developmental competence of each diameter group. At the same time, nuclear stage was assessed from a sample of oocytes of each diameter group before and after IVM. MPF subunits expression was studied by RT-PCR and Western blot for $p34^{cdc2}$. In addition, the protein and RNA stored were quantified in each diameter group by the Lowry method modified and by spectrophotometry, respectively. Results showed that, at collection time, most of the oocytes had already resumed meiosis, and this proportion was higher with increasing oocyte diameter. Moreover, the biggest oocytes reached nuclear maturation after IVM in a higher percentage, they showed higher percentage of normal fertilization, cleavage after 48 h post-insemination, and they produced the higher blastocyst rate. Our results showed that the largest oocytes, that were the most competent ones, did not differ from the rest of the groups in terms of $p34^{cdc2}$ RNA and mRNA, Cyclin B1 mRNA and total protein before and after IVM; however, we observed higher levels of total stored RNA and Cyclin B1 after IVM, higher level of $p34^{cdc2}$ protein and of MPF activity. Consequently, our results indicated that oocyte developmental competence was related to MPF, suggesting a role of this factor in cytoplasmic maturation.

The third work was arisen due to the results obtained in the first work. It surprised to us to observe that most of oocytes had resumed meiosis at collection time. Oocytes can resume meiosis inside the follicle as a consequence of the LH hormonal stimulus, prior ovulation, or due to a degeneration process or atresia. Our works were performed in prepubertal females, which were hormonally immature, so we raised the possibility that we were using oocytes suffering a process of atresia, which probably could impair our embryonic development results. The third study was conducted in the University of Gent, where they used techniques of detection of apoptosis in a routine way, and we used bovine oocytes due to the impossibility to obtain caprine oocytes. The aim of this study was to learn the techniques of

Annexin V staining and TUNEL by the accomplishment of a study of apoptosis in bovine oocytes, cumulus cells and blastocysts depending on oocyte diameter. For this purpose, oocytes obtained from the slaughterhouse were classified in three groups of size: <110 µm, 110-120 µm y >120 µm (Fair et al., 1995). Oocytes were matured and fertilized *in vitro*, and the resulting embryos were culture for additional 6 days. A sample of oocytes and cumulus cells were used to assessed apoptosis before and after IVM by means of Annexin V staining and TUNEL. The rest of the oocytes kept on developing, and the resulting blastocysts were also evaluated with the same techniques. In this study, oocyte developmental competence was achieved in oocytes higher than 110 µm. Our results showed that the percentage of immature oocytes showing signs of apoptosis decreased in oocytes > 110 µm. In addition, the proportion of apoptotic oocytes was reduced clearly during IVM. On the contrary, apoptosis in cumulus cells increased during maturation. Finally, the blastocysts obtained showed less incidence of late apoptosis in the biggest oocytes when evaluated by means of Annexin V, but not by means of TUNEL. From these results we could conclude that differences observed in different diameter groups in terms of embryonic development could not be explained only because a differential incidence of apoptosis among diameter groups.

Finally, the purpose of the fourth and fifth studies was to evaluate apoptosis in goat oocytes of different diameter by means of the two techniques used in the previous work. We wanted to introduce a new parameter in these studies, in addition to oocyte diameter, and so we classified the oocytes by morphological criteria as well: the ones that did not show any sign of atresia, that is, oocytes that had compact cumulus cells layers and homogeneous cytoplasm, and the ones that showed early signs of atresia, that is, oocytes with heterogeneous cytoplasm and/or cumulus cells with initial expansion. We believed that it would be more interesting to introduce the morphological classification because it could be use as a predictive parameter of the quality of the resulting embryos. For that purpose, oocytes were classified by diameter following the criteria explained in the first work, and by morphology following the criteria explained before.

Like in the previous study, apoptosis was evaluated in oocytes and cumulus cells before and after in vitro maturation, and in the blastocysts obtained. Like in bovine, we also observed in this study a decrease of apoptosis in oocytes and an increase in cumulus cells during IVM. In general, it was observed that the biggest oocytes with healthy morphology showed fewer incidences of apoptosis, although the results obtained differed depending on the technique used. Finally, we could observe that oocyte developmental competence did not only depend on oocyte diameter, but also in COC morphology. Consequently, we could conclude that apoptosis can affect oocyte developmental competence, but not blastocyst quality.

# 1. INTRODUCCIÓN

Durante los últimos años ha existido un interés creciente por las técnicas de reproducción asistida, no sólo en su aplicación clínica en humanos, sino también en el campo de la producción animal. El avance logrado en las técnicas de maduración *in vitro* (MIV), fecundación *in vitro* (FIV) y cultivo *in vitro* de embriones pre-implantacionales (CIV) ha permitido el desarrollo de otras tecnologías, como la obtención de células madre, el sexaje de embriones, la transgénesis o la clonación. La obtención de un mayor número de embriones a menor coste mediante la producción *in vitro* de embriones (PIV), en comparación con la técnica MOET (*Multiovulation and Embryo Transfer*), ha hecho posible el avance de las nuevas tecnologías anteriormente mencionadas. Por otro lado también ha permitido la recuperación de especies en peligro de extinción, la reproducción de animales muertos, acortar el intervalo generacional (de interés en programas de mejora genética) con el uso de animales prepúberes como donantes de ovocitos, etc. En general, la mayoría de los estudios sobre MIV, FIV y CIV realizados hasta el momento en animales de producción se han centrado en el bovino. Sin embargo, nuestro laboratorio ha optado por el caprino como modelo experimental, ya que presenta diversas ventajas frente al bovino: mayor manejabilidad, manutención menos costosa, período de gestación más corto y mayor prolificidad. Además, la posibilidad de obtener productos de interés farmacológico e industrial sintetizados en glándula mamaria posibilita que la cabra presente un alto interés en el campo de la transgénesis.

Los ovarios de cabras sacrificadas en matadero se ha convertido en la fuente más importante de ovocitos para la producción *in vitro* de embriones, básicamente debido a que se obtienen un gran número de ovocitos a un coste mucho menor que si se utilizaran animales vivos como donantes de ovocitos. Sin embargo, la gran mayoría de cabras sacrificadas para el consumo en nuestro país son prepúberes, de alrededor 1-2 meses de edad. Obtener los ovocitos de animales prepúberes supone una desventaja respecto a las adultas

en cuanto a la eficiencia en la producción de blastocistos (Armstrong, 2001); no obstante, la cantidad de ovocitos obtenidos por ovario en animales prepúberes es mayor (Baldassarre et al., 1997). Además producir embriones y descendencia de animales prepúberes intensifica la respuesta a la selección genética al acortar el intervalo generacional.

El trabajo de esta tesis es la continuación del trabajo que viene desarrollándose en nuestro laboratorio desde los años 90. Los primeros estudios realizados comprobaron que los ovocitos obtenidos de cabras prepúberes eran capaces de madurar *in vitro* (Martino et al., 1994), ser fecundados (Martino et al., 1995) y desarrollarse hasta blastocisto (Mogas et al., 1997). Sin embargo, también se observaron numerosas anomalías en la fecundación, como la no descondensación de la cabeza del espermatozoide o la polispermia (Martino et al., 1995; Mogas et al., 1997), y en el desarrollo embrionario, como la parada en el desarrollo en el momento de activación del genoma embrionario (Izquierdo et al., 1999) o una alta incidencia de haploidía (Villamediana et al., 2001). Todos estos estudios indicaban que las anomalías detectadas eran consecuencia de una deficiente maduración citoplasmática de los ovocitos utilizados, de modo que los estudios posteriores trataron de mejorarla mediante la selección de los ovocitos con *Brilliant Cresyl Blue* (Rodríguez-González et al., 2003a; Rodríguez-González et al., 2002; Urdaneta et al., 2003b), la adición de compuestos tioles en los medios de cultivo (Mayor et al., 2001; Rodríguez-González et al., 2003a; Rodríguez-González et al., 2003b; Urdaneta et al., 2003a; Urdaneta et al., 2004), o la premaduración (Jiménez-Macedo et al., 2006). Estos estudios permitieron incrementar el porcentaje de blastocistos obtenidos, aunque este porcentaje es más bajo que el obtenido con ovocitos de cabras adultas. El siguiente paso, englobado en el trabajo presentado en esta tesis, ha sido el estudio de la calidad ovocitaria a través de la búsqueda de marcadores moleculares que nos permitieran la selección únicamente de los ovocitos capaces de desarrollarse hasta el estadío de blastocisto, ya que la calidad ovocitaria es el principal factor que determina el éxito del desarrollo embrionario (Rizos et al., 2002).

Numerosos estudios han centrado sus esfuerzos en la búsqueda de marcadores de calidad ovocitaria, tales como el contenido mitocondrial del ovocito (Santos et al., 2006), la cantidad relativa de transcritos específicos en el ovocito (De Sousa et al., 1998; Watson et al., 2000) o en el embrión, la actividad transcripcional (Bilodeau-Goeseels y Panich, 2002) o la incidencia de apoptosis en ovocitos (Liu et al., 2000; Yang y Rajamahendran, 2002; Yuan et al., 2005), embriones (Liu et al., 2000; Pomar et al., 2005; Yang y Rajamahendran, 2002) o células del cumulus (Corn et al., 2005; Lee et al., 2001; Yuan et al., 2005; Zeuner et al., 2003). Sin embargo, la mayoría de marcadores se han estudiado mediante técnicas invasivas, de modo que el ovocito no puede utilizarse para su desarrollo posterior. Como consecuencia, es importante relacionar estos marcadores con características no invasivas (ej: morfología del cumulus, diámetro folicular, diámetro ovocitario). Existen varios estudios que relacionan el diámetro del folículo con la competencia de su ovocito para desarrollarse hasta blastocisto (bovino: Furher et al., 1989; caprino: Crozet et al., 1995; porcino: Marchal et al., 2002); de esta forma utilizan este criterio folicular, junto con la técnica de la aspiración, para seleccionar los ovocitos que se utilizarán en el proceso de producción *in vitro* de embriones. En ovarios de cabras prepúberes la textura del tejido y el tamaño de los folículos hacen impracticable este método de selección ovocitaria. El diámetro folicular ha sido relacionado de forma positiva con el diámetro ovocitario en bovino (Arlotto et al., 1996) y caprino (Crozet et al., 2000; de Smedt et al., 1994). Consecuentemente, en el presente trabajo de investigación se escogió el uso del diámetro ovocitario como indicador no invasivo.

Teniendo en cuenta las consideraciones anteriores, el objetivo de esta tesis fue el estudio de características moleculares en ovocitos de diferente tamaño que nos permitieran seleccionar aquéllos con mayor capacidad para desarrollarse hasta el estadío de blastocisto. Los posibles marcadores seleccionados para el estudio fueron:

**a)** La expresión a nivel transcripcional y traduccional del *Maturation Promoting Factor* (MPF), así como su actividad. El MPF es un heterodímero compuesto por la $p34^{cdc2}$ y la Ciclina B1, y se considera el principal regulador de la maduración nuclear del ovocito y un posible candidato a regular la maduración citoplasmática (Naito et al., 1992). La acumulación de una cantidad umbral de alguno de sus componentes ha sido descrita como el factor limitante para la reanudación meiótica en el ovocito ($p34^{cdc2}$: Chesnel y Eppig, 1995; De Vantéry et al., 1996; de Vantery et al., 1997; Dedieu et al., 1998; Mitra y Schultz, 1996; Ciclina B1: Levesque yd Sirard, 1996; Sun et al., 2001). Por lo tanto, también puede jugar un papel importante en la adquisición de competencia ovocitaria para el desarrollo (Vigneron et al., 2004).

**b)** la incidencia de apoptosis en ovocitos y células del cumulus dependiendo, no sólo del diámetro ovocitario, sino también en la morfología del complejo cumulus-ovocito (COC). El uso de ovarios procedentes de matadero supone obtener una población ovocitaria muy heterogénea, donde podemos encontrar diferentes grados de desarrollo y de atresia. De hecho, el 85% de los folículos presentes en un ovario están sufriendo atresia (Kruip y Dieleman, 1982). Por lo tanto, era necesario establecer la incidencia de la apoptosis en COC de cabras prepúberes y de qué modo podía influenciar el desarrollo embrionario. Para llevar a cabo este estudio se realizó un trabajo previo con ovocitos de vaca, con la finalidad de poner a punto los métodos que se iban a utilizar posteriormente con ovocitos de cabras prepúberes. A su vez, este estudio también nos sirvió como comparativa entre los ovocitos caprinos y bovinos.

# 2. REVISIÓN BIBLIOGRÁFICA

## 2.1. MADURACIÓN DEL OVOCITO

Cuando los ovocitos se forman, durante el desarrollo fetal de la hembra, quedan parados en la profase de la primera división meiótica después de sufrir una serie de divisiones mitóticas. En este estado los ovocitos no son capaces de ser fecundados y dar lugar a un embrión viable; es necesario que el ovocito crezca, junto con el folículo, y que se produzcan una serie de cambios morfológicos, ultraestructurales y moleculares que dotarán al ovocito de capacidad para finalizar la meiosis, originando una célula haploide, ser fecundado y dar lugar a un embrión viable. Este conjunto de cambios se conoce como maduración ovocitaria, que engloba dos procesos: la maduración nuclear o meiótica y la maduración citoplasmática.

### 2.1.1. Maduración nuclear o meiótica

La maduración nuclear en el ovocito es el proceso que tiene lugar desde la reanudación de la primera división meiótica hasta la parada en metafase de la segunda división meiótica. El ovocito solamente podrá completar la meiosis cuando se dé su reactivación mediante la fecundación. La maduración nuclear *in vivo* se inicia con el pico de la hormona luteinizante (LH), cuando el ovocito ya ha completado su fase de crecimiento. *In vitro*, los ovocitos también son capaces de madurar cuando se les libera de su folículo (Pincus y Enzmann, 1935; revisado por Ponderato et al., 2001); en este caso es necesario que el ovocito haya alcanzado el 80-90% de su tamaño final para que pueda reanudar la meiosis.

Al recibir el estímulo pre-ovulatorio de la LH se produce la activación de una cascada de reacciones en las que intervienen numerosas proteínas con

actividad kinasa que conducirán a la ruptura de la vesícula germinal (GVBD = *Germinal vesicle breakdown*). Los nucleolos desaparecen, se produce la polimerización de los microtúbulos, y los cromosomas se condensan y se alinean en la placa metafásica (Metafase I). Seguidamente se separan los pares de cromosomas homólogos en una división asimétrica que origina dos células de diferente tamaño: el primer corpúsculo polar (PBI), y el ovocito secundario. Al iniciarse la meiosis II el ovocito no entra en profase y los cromosomas permanecen condensados, en un período conocido como interkinesis. El ovocito secundario, que ahora se encuentra en estadío de metafase II, sufre una nueva parada meiótica, y es en este estadío cuando, en la mayoría de mamíferos, el ovocito es ovulado. Sólo se completará la meiosis en caso de que el ovocito se active por la penetración de un espermatozoide, extruyendo al final el segundo corpúsculo polar (PBII).

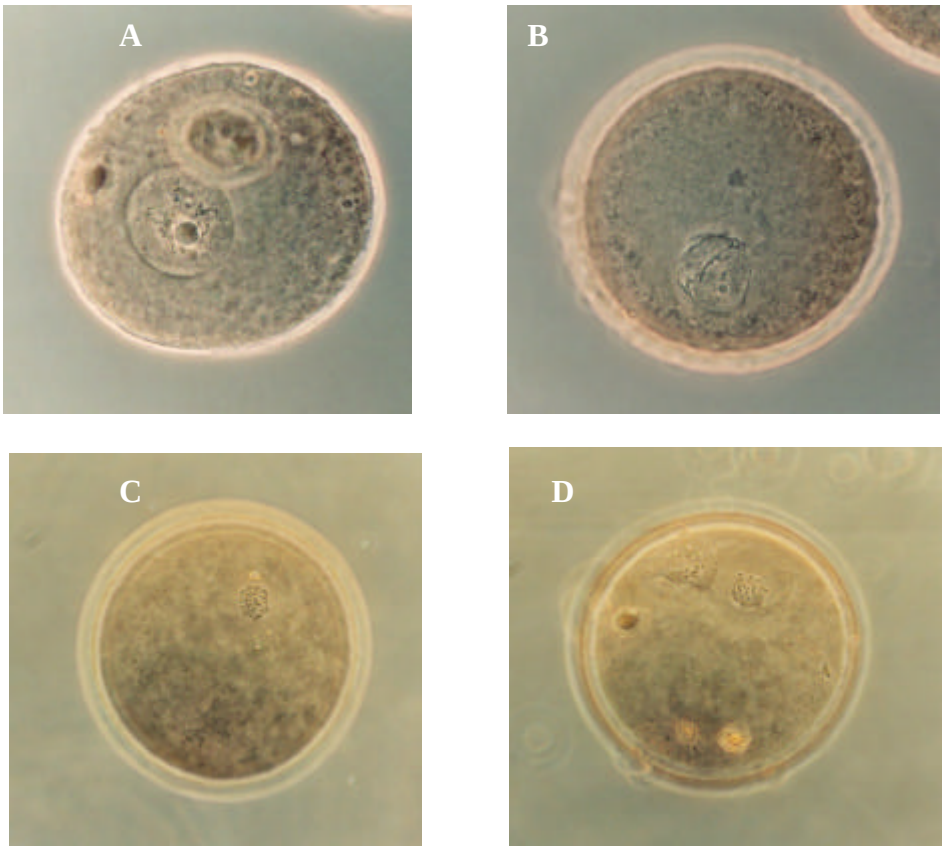


Figura 1. Diferentes estadios meióticos en el ovocito (tinción con lacmoide): A) Vesícula germinal (GV); B) Ruptura de la vesícula germinal (GVBD); C) Metafase I (MI); D) Metafase II (MII)

### 2.1.2. Maduración citoplasmática

La maduración citoplasmática comprende todos los procesos que tienen lugar en el citoplasma y que van a posibilitar que el ovocito sea capaz de descondensar la cabeza del espermatozoide tras la fecundación y mantener el posterior desarrollo embrionario (Mermillod et al., 1999; Prather y Day, 1998). A diferencia de la maduración nuclear, que puede ser fácilmente observada con el microscopio óptico, la evaluación de la maduración citoplasmática es mucho más complicada, y debería valorarse según la capacidad del ovocito para dar lugar a un embrión viable, capaz de desarrollarse y dar lugar al nacimiento de un animal vivo y sano. Sin embargo, debido a la dificultad de llevar a cabo estos procesos a nivel experimental, generalmente se evalúa la maduración citoplasmática de un ovocito mediante su capacidad para llegar al estadío embrionario de blastocisto.

La acumulación de ARNm y proteínas en el citoplasma durante el crecimiento del ovocito son esenciales para que éste puede mantener el desarrollo embrionario hasta que se produzca la activación del genoma del embrión (Bachvarova, 1992), que tiene lugar en embriones de 2 células en ratón, de 4 células en cerdos, de 4-8 células en humanos, de 8 células en conejo, y embriones de 8-16 células en vacas y ovejas (Telford et al., 1990). En la especie caprina, igual que en los rumiantes citados, la activación del genoma embrionario se produce a las 8-16 células. El almacenamiento del ARNm en el ovocito se produce mayoritariamente por poliadenilación citoplasmática selectiva (Gandolfi y Gandolfi, 2001), ya que se ha observado que las colas poli-A son cortas cuando el ARNm materno se almacena, y se alargan para que el ARNm sea reclutado para la traducción (Brevini-Gandolfi et al., 1999; Huarte et al., 1992; Temeles y Schultz, 1997). La regulación de

la transcripción del ARNm almacenado también se produce por enmascaramiento de elementos específicos de determinados ARNm (Gandolfi y Gandolfi, 2001), impidiendo así su traducción a proteínas. El ARN que se transcribe durante la maduración citoplasmática es muy estable, con una vida media aproximada de 28 días (Wassarman et al., 1996).

Al mismo tiempo, a medida que el ovocito va creciendo también se produce la redistribución y maduración de orgánulos citoplasmáticos (Caralco, 1995; Ducibella et al., 1994; Hyttel et al., 1986). Las mitocondrias, que se encuentran en el centro del ovocito al inicio de su crecimiento, migran hacia la periferia a medida que aumenta el tamaño ovocitario (Cran, 1985; Fair et al., 1995). Los gránulos corticales, importantes para prevenir fenómenos de polispermia durante la fecundación (Guraya, 1982), migran del centro hasta situarse por debajo de la membrana citoplasmática del ovocito (Assey et al., 1994; Fair et al., 1995). El número de aparatos de Golgi, precursores de los gránulos corticales, también aumenta con el diámetro del ovocito (Fair et al., 1995). El Retículo Endoplasmático se distribuye de forma dispersa por el citoplasma, formando sacos densos que avanzan hacia la periferia con el crecimiento ovocitario.

Pocos días antes del pico de LH se dan en el ovocito los últimos cambios a nivel citoplasmático que van a conducir a su "capacitación", es decir, van a dotarle de competencia para madurar y ser fecundado correctamente, y mantener el desarrollo embrionario posterior (Hyttel et al., 1997). Se produce una disminución del aparato de Golgi, y un reagrupamiento de bs gránulos corticales (Cran, 1985). Se inhibe la síntesis de ARN y proteínas, que era muy activa durante el crecimiento del ovocito, mediante la condensación del nucleolo y la eliminación de ribosomas (Fair et al., 1995; Hyttel et al., 1986, 1989). Justo antes de la ovulación aparece entre el ovocito y la zona pelúcida el espacio perivitelino, que aumentará de volumen con el tiempo.

### 2.1.3. Papel del cumulus oophorus en la maduración del ovocito

La unión de las células del cumulus al ovocito durante la maduración es importante para completar con éxito no sólo la maduración, sino también la fecundación y el posterior desarrollo embrionario (Atef et al., 2005; Wongsrikeao et al., 2005). Las células del cumulus se comunican entre ellas y con el ovocito mediante uniones tipo gap (Eppig, 1982; Furger et al., 1996; Moor et al., 1980), lo que permite el intercambio de moléculas entre los dos tipos celulares, produciéndose una regulación cumulus-ovocito recíproca (Heikinheimo y Gibbons, 1998).

Las células del cumulus juegan un papel importante en la maduración del ovocito mediante la regulación de varios procesos:

a) el mantenimiento del ovocito en parada meiótica: la transferencia directa de sustancias desde las células del cumulus al ovocito mantienen a éste en parada meiótica. Hay varias sustancias que se consideran inhibidoras de la meiosis, como purinas (Eppig et al., 1985) y el AMPc (*cyclic Adenosine monophosphate*) (Kumar y Gilula, 1996).
b) La reanudación de la meiosis: Una vez el ovocito ha completado su crecimiento, la reanudación de la meiosis *in vivo* se produce por la pérdida de uniones gap entre el cumulus y el ovocito a consecuencia de la expansión de las células del cumulus (Wert y Larsen, 1989) inducida por el pico de LH que tiene lugar antes de la ovulación. La pérdida de las uniones impide el paso de sustancias inhibidoras de la meiosis al ovocito.
c) la maduración citoplasmática: la presencia de células del cumulus durante la maduración favorece la posterior descondensación de la cabeza del espermatozoide y su transformación a pronúcleo masculino, mediante el aumento de la concentración intraovocitaria de glutatión (Calvin et al., 1986; Perreault et al., 1988; Yoshida et al., 1993), además de favorecer la fecundación monospérmica y el desarrollo embrionario (ovejas: Staigmiller y Moor, 1984; ratas:

Vanderhyden y Armstrong, 1989; vacas: Chian et al., 1994; cerdos: Yamauchi y Nagai, 1999). Esta acción de las células del cumulus se produce mediante glicosaminoglicanos, hormonas esteroideas y otros factores que promueven la maduración citoplasmática en el ovocito (Brower y Schultz, 1982; Danforth, 1995; Dode y Graves, 2002; Yamauchi y Nagai, 1999). Además las células del cumulus estabilizan los gránulos corticales, impidiendo que se produzca una migración prematura o una exocitosis parcial (Galeati et al., 1991).

d) Protección del ovocito: las células del cumulus también intervienen en la protección del ovocito promoviendo la reducción de la cistina presente en el medio a cisteína, y la captura de cisteína por parte del ovocito (Takahashi et al., 1993) de modo que aumenta la concentración intraovocitaria de glutatión. El glutatión mantiene del estado redox de las células, protegiendo al ovocito del estrés oxidativo (Tatemoto et al., 2000).

### 2.1.4. Regulación de la maduración ovocitaria

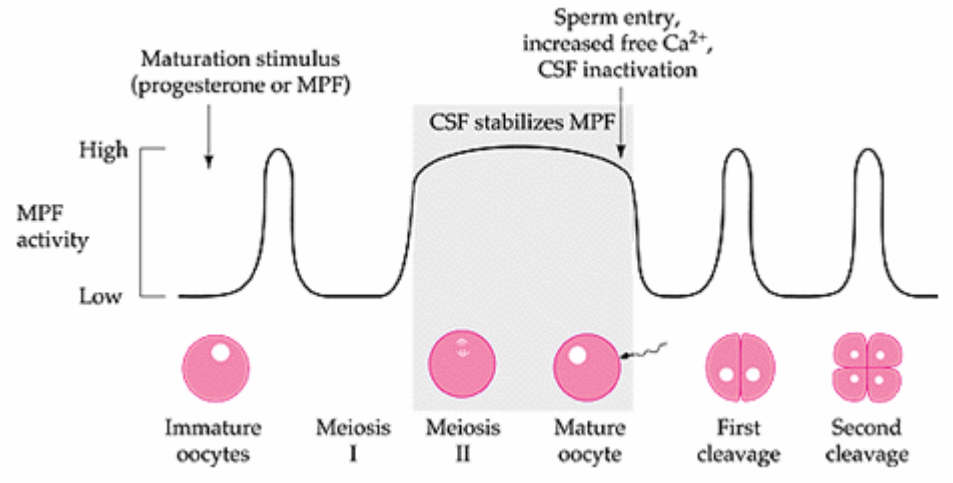
El AMPc es continuamente transferido desde las células del cumulus a los ovocitos en crecimiento para mantener su parada meiótica (Dekel, 1988). Está ampliamente aceptado que el AMPc es la señal inhibidora de la meiosis ovocitaria. Concentraciones altas de AMPc en el ovocito promueve la actividad catalítica de PKA (*AMPc dependent protein kinase*), que impide la activación del MPF *(Maturation Promoting Factor,* también conocido como *M-phase promoting factor)* a través de la inactivación de la fosfatasa Cdc25 y de la inhibición de la síntesis de Ciclina B1 (revisado por Dekel, 2005). A su vez, la localización subcelular de PKA está regulada por APAK (*A kinase anchoring proteins*), que actúa como un mecanismo complementario para la regulación de la actividad de PKA y un mejor control de la parada meiótica (revisado por Dekel, 2005). Cuando se produce el pico de LH se genera una disminución de la cantidad de AMPc que llega al ovocito desde las células del cumulus, debido a una pérdida de conexiones célula-célula. La disminución de la concentración

ovocitaria de AMPc, junto con la translocación de la PKA de su lugar de acción, inhibe la actividad catalítica de PKA. A consecuencia, se produce la activación de la fosfatasa Cdc25 mediante fosforilación. Esta fosfatasa provocará la activación del MPF que dará lugar a la reanudación de la meiosis y la progresión hasta MII.

El MPF fue descrito por primera vez por Masui y Markert (1971) en ovocitos de anfibios, y posteriormente ha sido descrito en numerosas especies. El MPF está compuesto por dos subunidades: la subunidad catalítica ($p34^{cdc2}$) que presenta actividad seronina/treonina kinasa, y la subunidad reguladora (Ciclina B1) (Gautier et al., 1990). Este heterodímero se forma inicialmente como un pre-MPF inactivo, en el cual la subunidad catalítica presenta los residuos Thr 161, Thr 14 y Tyr 15 fosforilados. La activación del complejo MPF se produce por la defosforilación de la Thr 14 y Tyr 15 mediada por la fosfatasa Cdc25 B (Gould y Nurse, 1989; revisado por Dekel, 2005), quien a su vez es fosforilada por el MPF, dando lugar a una retro-alimentación positiva requerida para una activación rápida del complejo (Hoffmann et al., 1993).

La actividad del MPF sigue un patrón oscilatorio, que ha sido demostrado en muchas especies: se produce un aumento de la actividad de MPF justo antes de la GVBD, alcanza un pico máximo en MI, disminuye su actividad durante la anafase-telofase para que puedan separarse los cromosomas, y aumenta de nuevo para alcanzar un nuevo pico máximo en estadío de MII (Ratón: Choi et al., 1991; Fulka et al., 1992; Cerdo: Naito y Toyoda, 1991; Bovino: Collas et al., 1993; Conejo: Jelinkova et al., 1994; Cabra: Dedieu et al., 1996). Entre la meiosis I y II, aunque la actividad del MPF disminuye, se mantiene a un nivel básico para impedir la replicación del ADN.

Figura 2. Representación esquemática de la actividad de MPF a lo largo de la maduración del ovocito, fecundación y primeros estadíos de desarrollo embrionario (www.med.yale.edu).




La activación del MPF da lugar a la ruptura de la envoltura nuclear (GVBD), la condensación de los cromosomas, su disposición en la placa metafásica y la poliadenilación del ARNm mos para promover su traducción. Niveles elevados de MPF activo inhiben la interfase y la extrusión del primer corpúsculo polar. La actividad de $p34^{cdc2}$ controla el complejo APC/C, que es el responsable de la degradación de la Ciclina B1, de manera que el MPF activo induce su propia inactivación a través de la degradación de la Ciclina B1 para salir de la primera división meiótica (Frank-Vaillant et al., 2001) y para que se libere el primer corpúsculo polar. Debe darse acumulación de Ciclina B1 para que aumente de nuevo la actividad de MPF. La actividad máxima de MPF se alcanza en MII, y sólo disminuirá en caso de que se produzca la fecundación del ovocito. La disminución de la actividad del MPF se produce por la disociación del heterodímero y la degradación proteosomal de la Ciclina B1 (Josefsberg et al., 2000).

Aunque el MPF ha sido ampliamente estudiado, se conocen pocos sustratos sobre los que actúa. La histona H1 es el clásico sustrato del MPF, y su fosforilación por parte del complejo da lugar a la condensación de los cromosomas. Las lamininas nucleares también son fosforiladas por el MPF, así

como la proteína kinasa MELK, que interviene en el control del ciclo celular, la proliferación celular y el *splicing* de ARNm (Badouel et al., 2006). Además, se ha sugerido una regulación por parte del MPF de la actividad de la pp60c-src y ARN polimerasa II, que conduciría a reorganizaciones del citoesqueleto y a la inhibición de la transcripción que tiene lugar durante la división celular, respectivamente (revisado por Heikinheimo y Gibbons, 1998).

El MPF se considera el principal regulador de la maduración nuclear, pero no es la única molécula que interviene en este proceso. Otros factores que juegan un papel importante son las proteínas ERK-1 y ERK-2 (*Extracellular signal-regulated kinases*), que forman parte de la familia de las MAPK (*Mitogen activated Protein kinase*) (Lazar et al., 2002). El MPF regula la poliadenilación del protooncogen mos, de modo que cuando el MPF está activo se produce síntesis de proteína mos. Mos da lugar a la activación de MEK Kinasa, quien a su vez regula la actividad de MEK, la proteína kinasa que activa la proteína MAPK. La expresión del protooncogen mos, y por lo tanto, la actividad de MAPK, están reguladas por las concentraciones intraovocitarias de AMPc (Lazar et al., 2002), a través de la actividad de MPF (Lazar et al., 2004). A su vez, la activación de MAPK también conduce a la activación del MPF, a través de la inhibición de la degradación de la Ciclina B1, lo que da lugar a la acumulación de la subunidad reguladora durante el paso de meiosis I a meiosis II, y mantiene los niveles de MPF elevados durante la parada meiótica en metafase II (revisado por Heikinheimo y Gibbons, 1998). Aunque se ha visto que en ovocitos de *Xenopus* la expresión de mos es necesaria para la reanudación de la meiosis (revisado por Roy et al., 1996), en algunos mamíferos se ha observado que la reanudación de la meiosis es un proceso independiente de MAPK (Lazar et al., 2002). La principal función de MAPK en el proceso meiótico es mantener la parada en estadío de metafase II (Colledge et al., 1994; Hashimoto et al., 1994) hasta que se produzca la fecundación.

Debido al importante papel que juega el MPF en la maduración nuclear del ovocito, numerosos autores han hipotetizado que la deficiencia en la síntesis de alguna de las dos subunidades del complejo, o de su actividad,

podían ser las causas de la incompetencia de los ovocitos para reanudar la meiosis. Se ha observado que las posibles causas de la incompetencia meiótica ovocitaria varía entre especies:

- En ratón, la adquisición de competencia meiótica se asocia en parte con la síntesis de $p34^{cdc2}$ (Chesnel y Eppig, 1995; de Vantéry et al., 1996, 1997; Mitra and Schultz, 1996), y no con la de Ciclina B1 (Chesnel y Eppig, 1995; de Vantéry et al., 1996).
- En cabras, mientras que la Ciclina B1 en forma de ARNm y proteína se detecta en ovocitos competentes e incompetentes (Hue et al., 1997), la $p34^{cdc2}$ sólo se encuentra en ovocitos meióticamente competentes (Dedieu et al., 1998). Esto indica que, en esta especie, la incapacidad de los ovocitos incompetentes para reanudar la meiosis podría deberse a la ausencia de $p34^{cdc2}$ en estos ovocitos, necesitándose de esta manera una concentración mínima de esta proteína para dar lugar a la ruptura de la vesícula germinal (Crozet et al., 2000; Dedieu et al., 1998). Además Dedieu et al. (1998) hipotetizan que la cantidad de $p34^{cdc2}$ en ovocitos parcialmente competentes no es suficiente para reanudar la meiosis espontáneamente en el momento adecuado, produciéndose un retraso en la maduración.
- En cerdos, al contrario, los 2 componentes del MPF se encuentran en los ovocitos inmaduros, sugiriendo que en esta especie la parada meiótica se controla por otros mecanismos (Christmann et al., 1994). Sin embargo, estudios posteriores han determinado que la síntesis de Ciclina B1 es necesaria para reanudar la meiosis (Sun et al., 2001).
- En bovino, la proteína Ciclina B1 no se detecta en el estadío de vesícula germinal (Levesque y Sirard, 1996) pero sí se detecta su ARNm, cuyos niveles disminuyen durante el crecimiento folicular (Robert et al., 2002). La reanudación de la meiosis en bovino se inicia con la acumulación de proteína ciclina B1 (Levesque y Sirard, 1996).

La regulación de la maduración citoplasmática, al contrario que la regulación de la maduración nuclear, es bastante desconocida; sin embargo, se cree que el MPF y la maduración citoplasmática podrían estar relacionados

de algún modo, ya que se ha observado que ovocitos con baja actividad MPF en metafase II son incapaces de formar el pronúcleo masculino después de la fecundación (Naito et al., 1992), debido a una incapacidad para eliminar la membrana nuclear del espermatozoide (Peter et al., 1990). Niveles bajos de MPF en la segunda parada meiótica no serían suficientes para activar la degradación de la Ciclina B1, y por lo tanto, la disociación del MPF que se produce tras la fecundación.

## 2.2. APOPTOSIS

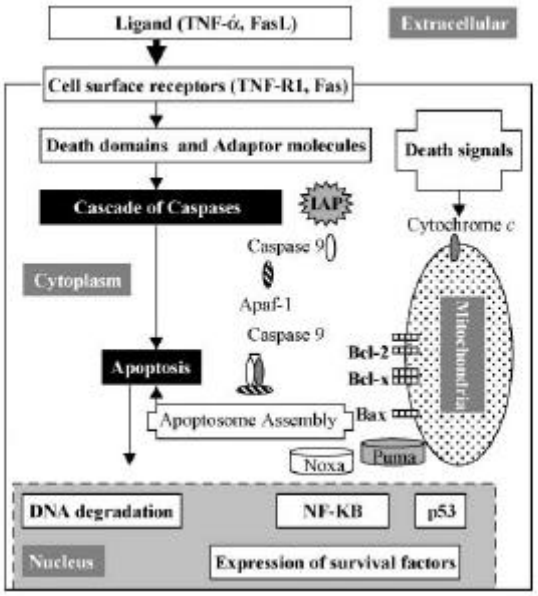
### 2.2.1. Conceptos generales

La apoptosis es el mecanismo que permite regular procesos biológicos, como la morfogénesis y la homeostasis tisular, mediante la eliminación de células defectuosas o que ya no son necesarias (Steller, 1995). Es un tipo de muerte celular programada totalmente controlada por la expresión de determinados genes, y caracterizada por una serie de cambios morfológicos y bioquímicos (Wyllie et al., 1980).

Existen 2 rutas principales de señalización de la apoptosis: la dependiente de receptor y la dependiente de mitocondria (Ashkenazi y Dixit, 1998; Green y Reed, 1998; Nagata, 1997; revisado por Hussein, 2005). En la primera, la unión de diferentes ligandos, como TNF (*tumor necrosis factor*), TRAIL (*TNF- related apoptosis-inducing ligand*), Fas o APO3L, a sus receptores desencadena una cascada de reacciones que tendrá como consecuencia final la activación de caspasas efectoras y la muerte de la célula. Esta ruta está regulada por Flip (*FLICE inhibitory protein*), que previene la activación de caspasas iniciadoras, y por IAP (*inhibitor of apoptosis*) (Hussein et al., 2003). En la ruta dependiente de mitocondria, factores inductores de la apoptosis, como la irradiación, la presencia o la ausencia de citokinas o determinados factores de crecimiento, inducen la desestabilización de la membrana mitocondrial y, por consiguiente, la liberación de citocromo-c al citosol, que se unirá al Apaf-1 (*apoptotic protease-activating factor 1*) (Robles et al.,

1999; Zou et al., 1997). Este complejo se une a la pro-caspasa-9, la activa y se inicia la activación de caspasas efectoras, como la caspasa-3 (Grutter, 2000; Wang, 2001), dando lugar a la muerte celular.

Figura 3. Representación de las dos rutas que intervienen en la activación y ejecución de la apoptosis (Hussein, 2005).



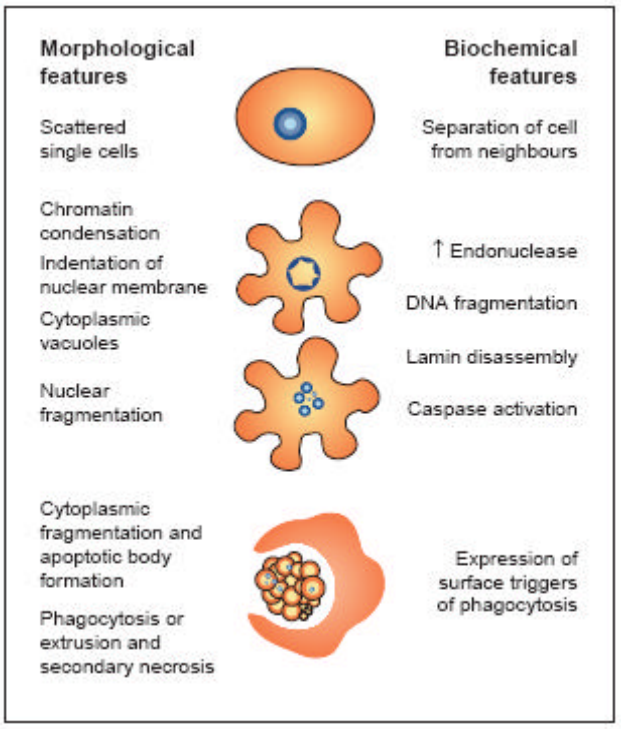

## 2.2.2. Métodos de detección de la apoptosis

### *2.2.2.1. Evaluación morfológica*

La apoptosis fue descrita por primera vez por Kerr et al. (1972), quienes describieron las distintas fases morfológicas que permiten distinguir una célula que está sufriendo un proceso apoptótico: inicialmente se produce la condensación nuclear debido a la redistribución de la cromatina junto a la membrana nuclear, el citoplasma se condensa dando lugar a la pérdida de volumen celular, y las membranas nuclear y citoplasmática pierden integridad. Seguidamente el ADN se fragmenta, y se forman unas vacuolas rodeadas de membrana que contienen parte del citoplasma, orgánulos celulares y fragmentos nucleares, conocidos como cuerpos apoptóticos o

picnóticos, que serán fagocitados por las células vecinas. La apoptosis, además, se caracteriza porque no lleva asociada una respuesta inflamatoria, al contrario que sucede con la necrosis (Kerr et al., 1994).

Figura 4. Representación esquemática de las principales características morfológicas y bioquímicas de la apoptosis. (Hardy, 1999)




Sin embargo, estudios posteriores observaron que algunas de las características morfológicas presentes en estadíos iniciales de la apoptosis también se observaban en el inicio de la necrosis (Columbano, 1995; Lemasters et al., 1998; Rosales-Torres et al., 2000; Zamai et al., 1996; Zamzami et al., 1997). Se ha postulado que la apoptosis y la necrosis se inician con una alteración de la integridad de la membrana mitocondrial (Columbano, 1995; Lemasters et al., 1998; Zamzami et al., 1997), pero el tipo de muerte celular que se llevará a cabo viene determinado según el contenido energético de la célula (Vayssiere et al., 1994; Zamzami et al., 1995): si los niveles de ATP son altos se desencadenará la apoptosis (Ankarcrona et al., 1995; Leist et al., 1997), mientras que si son bajos se producirá la necrosis (Ankarcrona et al., 1995). Además, la apoptosis se inicia antes de que las señales morfológicas de degeneración sean visibles (Asselin et al., 2000). Todo

ello, unido a la dificultad de detectar células apoptóticas mediante un microscopio óptico, hace necesario combinar la evaluación morfológica de las células con otras técnicas para poder identificar la apoptosis.

*2.2.2.2. Expresión de proteínas*

En la regulación y ejecución de la apoptosis intervienen numerosas proteínas y factores, cuya expresión puede servir para evaluar la incidencia de apoptosis en una célula determinada. Entre estos factores podemos destacar los siguientes:

- **Familia Bcl-2** (*B-Cell lymphoma-leukemia-2*): contiene proteínas inductoras (Bax, Bak, Bok) e inhibidoras de la apoptosis (Bcl-2, Bcl-$x_L$) (revisado por Guthrie et al., 2000). La proteína Bcl-2 (anti-apoptótica) previene la apoptosis manteniendo la integridad de la membrana mitocondrial (Yang et al., 1997). En cambio, cuando Bax (pro-apoptótica) se sobre-expresa, forma heterodímeros con Bcl-2, contrarrestando de esa manera los efectos de esta molécula en la supervivencia de la célula (Oltvai et al., 1993). Por lo tanto, la ratio Bcl-2/Bax es determinante en la supervivencia o muerte de la célula (Oltvai et al., 1993).

- **Citocromo c:** la activación de la apoptosis compromete la integridad de la membrana mitocondrial, dando lugar a la liberación al citosol del citocromo c, que se encontraba en el interior de la mitocondria (Kelekar y Thompson, 1998).
- **Caspasas**: son cisteína-proteasas que intervienen en el proceso de apoptosis. Encontramos dos tipos de caspasas, las iniciadoras (caspasa-2, caspasa-8, caspasa-9, caspasa-10) que responden a un estímulo proapoptótico dando lugar a la activación del otro tipo de caspasas, las efectoras (caspasa-3, caspasa-6, caspasa-7), que se encargan de ejecutar la muerte celular.

- **Proteína p53**: es un factor de transcripción que controla la proliferación celular. Esta proteína regula la apoptosis a nivel genético cuando hay daño irreparable en el ADN de la célula, induciendo la transcripción de Bax e inhibiendo la transcripción de Bcl-2 y promoviendo así su muerte (Ding y Fisher, 1998; Evan y Littlewood, 1998; Ko y Prives, 1996).

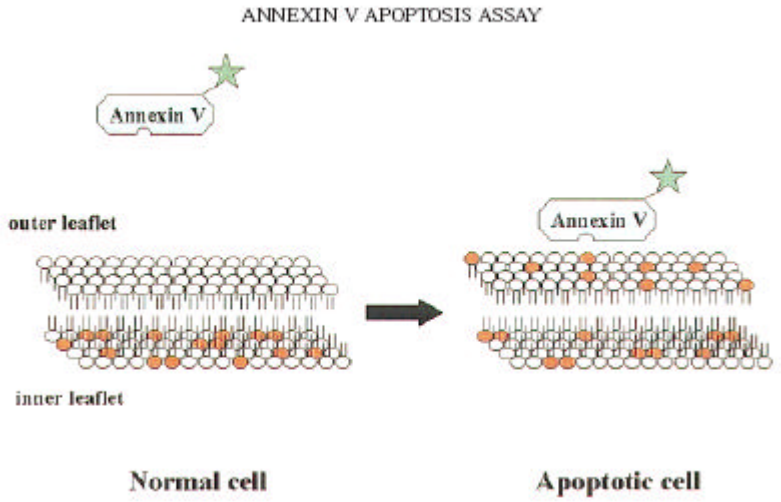
*2.2.2.3. Tinción con Annexin V*

Las células viables mantienen una asimetría entre la cara externa e interna de la membrana plasmática (Bretscher, 1972), de modo que la fosfatidilcolina y la esfingomielina se encuentran en la cara externa, mientras que la fosfatidilserina y la fosfatidiletanolamina se observan sólo en la cara interna. Esta asimetría de membrana se mantiene por la acción de unas proteínas denominada flipasas (Higgins, 1994; revisado por Diaz y Schroit, 1996). Las células tienen la habilidad de translocar la fosfatidilserina a la cara externa de la membrana citoplasmática en determinadas condiciones (Fadok et al., 1992, 1993), sirviendo como diana de reconocimiento específica para los macrófagos que deben fagocitar a las células en degeneración. Durante la apoptosis se produce la activación de unas proteasas que degradan la fodrina, responsable del ancoraje de la fosfatidilserina en la cara interna de la membrana celular (revisado por van Engeland et al., 1998). De este modo, la detección de la fosfatidilserina en la cara externa de la membrana puede servir como indicador de apoptosis.

La annexin V es una molécula que se une específicamente a la fosfatidilserina en presencia de calcio (Andree et al., 1990; Tait et al., 1989). La combinación de la annexin V con biotina o fluorocromos permite su detección mediante reacciones colorimétricas, citometría de flujo o microscopía de fluorescencia, lo que facilita la detección de células apoptóticas. La annexin V no es capaz de atravesar la membrana plasmática en las células viables; sin embargo, en células muertas, que han perdido la

integridad de membrana, la annexin V puede atravesar la bicapa lipídica y unirse a la fosfatidilserina que se encuentra en la cara interna de la membrana. Para discriminar entre células muertas y células apoptóticas, es necesario combinar la técnica con una tinción de ADN impermeable a la membrana, como el yoduro de propidio (revisado por van Engeland et al., 1998). Esta molécula sólo podrá entrar en las células cuando su membrana haya perdido su integridad, que se da cuando la célula está muerta o en las últimas fases de apoptosis (Collins et al., 1997; Martin et al., 1995). La pérdida de asimetría de membrana y, en consecuencia, la exposición de fosfatidilserina en la cara externa sucede en estadíos iniciales de la apoptosis, que se inicia como consecuencia de la activación de la cascada de caspasas pero antes de que se produzca la fragmentación del ADN (revisado por van Engeland et al., 1998).

Figura 5. Representación esquemática de la pérdida de asimetría de la membrana plasmática durante la apoptosis, y su detección mediante annexin V (van Engeland et al., 1998).




*2.2.2.4. Visualización de escalera de ADN*

Una de las características bioquímicas asociadas a la apoptosis es la pérdida de integridad del ADN mediante su fragmentación por la acción de

una endonucleasa (Williams et al., 1974). La fragmentación se produce a nivel internucleosomal, de modo que los fragmentos de ADN que se originan tienen una longitud múltiple de 180-200 pb. Este patrón en la fragmentación de ADN puede ser fácilmente evaluado mediante electroforesis en un gel de agarosa (Wyllie et al., 1980) a través de la aparición de "escaleras de ADN" correspondientes a los oligonucleosomas. Sin embargo, es posible que se produzca apoptosis sin la formación de estas "escaleras de ADN" (Cohen et al., 1992; Collins et al., 1992), lo que limitaría la eficiencia de esta técnica para la identificación de la apoptosis. Por otro lado, la necesidad de disponer de gran cantidad de ADN para poder visualizarlo en un gel de agarosa hace imposible el análisis cuando el número de células es limitado, como en los embriones.

*2.2.2.5. TUNEL (Tdt-mediated dUTP nick-end labelling)*

La técnica de TUNEL fue desarrollada por Gavrieli et al. (1992) para poder identificar células apoptóticas. Se basa en el uso de una enzima, la *terminal deoxynucleotidyl transferase* (Tdt), que cataliza la adición de dUTPs marcados con biotina o fluorocromos a los extremos 3'-OH presentes en el ADN fragmentado. De ese modo, el núcleo de las células apoptóticas podrá visualizarse mediante una reacción colorimétrica, o mediante el microscopio de fluorescencia. La ventaja de esta técnica respecto a la anterior es que permite el análisis de células individualizadas, de modo que se puede localizar y cuantificar el porcentaje de células que están sufriendo apoptosis.

La técnica de TUNEL tiene la desventaja de que no permite distinguir las células apoptóticas de las necróticas, debido a que en los dos procesos se produce fragmentación de ADN, lo que hace necesaria combinar el análisis con algunos de los métodos descritos anteriormente, como la Annexin-V o la detección de actividad caspasa. Aún así, es una técnica validada y ha sido utilizada en la detección de apoptosis en ovocitos y embriones de numerosas especies (bovino: Warzych et al., 2006; Yuan et al., 2005; Zeuner et al., 2003;

murino: Brison and Schultz, 1997; humano: Corn et al., 2005; Jurisicova et al., 1998; porcino: Kidson et al., 2004).

### 2.2.3. Apoptosis en el ovario

#### *2.2.3.1. En el período pre-natal*

En la mayoría de vertebrados las células germinales primordiales llegan a la cresta genital donde, después de sufrir una serie de divisiones mitóticas, inician la meiosis y quedan parados en la primera profase. Durante este período, la degeneración de las ovogonias se produce básicamente en dos etapas: en el estadío de paquiteno de la meiosis y durante la formación de los folículos primordiales (Baker, 1963). Como resultado de esta muerte celular sólo alrededor del 20-30% de las ovogonias son incluidas en un folículo primordial (revisado por Tilly, 1996; revisado por Lévy, 2005), y constituirán la reserva ovárica de la hembra. Aunque se desconoce cuáles son los factores que determinan la supervivencia o muerte de las ovogonias, parece ser que el SCF (*Stem cell growth factor*), el LIF (*Leukaemia inhibitory factor*) y la activación del receptor de ácido retinoico podrían jugar un papel importante (revisado por Tilly, 1996). Por otro lado, parece ser que la calidad de las mitocondrias del ovocito también tendrían un papel importante en la decisión de supervivencia o muerte del ovocito durante este período (revisado por Hussein, 2005).

#### *2.2.3.2. En el período post-natal*

Una vez establecido el pool de folículos primordiales, la atresia ovocitaria después del nacimiento se puede producir a consecuencia de la degeneración de los folículos que no se seleccionan para ser ovulados (Hirshfield, 1991; Tsafriri y Braw, 1984), o bien debido a la desaparición de folículos primordiales de la reserva o de folículos preantrales (revisado por

Reynaud y Driancourt, 2000). Mientras que se considera que la atresia folicular en folículos antrales y pre-antrales se inicia con la apoptosis de células de la granulosa, la causa de atresia en folículos primordiales es la muerte del ovocito (revisado por Reynaud y Driancourt, 2000). Parece ser que esta diferencia podría deberse a la desaparición de ADNasa I en el ovocito (Boone y Tsang, 1997), que coincide con la aparición del antro en el folículo.

En los animales que han alcanzado la pubertad, en cada ciclo se produce el reclutamiento folicular, pero sólo uno de los folículos reclutados se selecciona para ser dominante, mientras que el resto degenera en un proceso conocido como atresia (Ginther et al., 2001). La atresia en la mayoría de folículos se da durante la última etapa del período preantral y la primera del período antral, cuando el crecimiento folicular todavía es dependiente de gonadotropinas (Dalin, 1987); revisado por Tilly, 1996), lo que sugiere una regulación hormonal de la apoptosis. La FSH y la LH inhiben la apoptosis que ocurre en las células de la granulosa de los folículos que degeneran tanto *in vivo* (Billig et al., 1994) como *in vitro* (Chun et al., 1994; Kaipia y Hsueh, 1997; Tilly y Tilly, 1995). En la mujer, cuando se inicia el ciclo menstrual los niveles de FSH son altos, lo que permite que algunos folículos empiecen a crecer. Entre estos folículos se encuentra el dominante, que produce gran cantidad de estrógenos que van a inhibir la secreción de FSH, provocando que el resto de folículos en crecimiento inicien la apoptosis (Hughes y Gorospe, 1991; Tilly et al., 1991; revisado por Hussein, 2005).

En hembras prepúberes, al igual que en las adultas, también se produce reclutamiento de folículos para su crecimiento, pero todos degeneran por atresia debido a la falta de la señal hormonal adecuada (Tilly y Tilly, 1995). En hembras de edad avanzada, en cambio, la apoptosis no sólo se encarga de la eliminación de los folículos reclutados que no llegan a ser pre-ovulatorios, sino también de la eliminación de ovocitos defectuosos debido a la edad materna (revisado por Lévy, 2005).

Los primeros signos de atresia en los folículos antrales es la degeneración de la células de la granulosa murales y su pérdida de actividad aromatasa (Irving-Rodgers et al., 2001) seguido de la hipertrofia de las células de la teca y la disminución de la producción de androsterona (Driancourt et al., 1998). Esta degeneración de las células de la granulosa se produce mediante la apoptosis (Hughes y Gorospe, 1991). Las células del cumulus y el ovocito son los últimos compartimentos de folículo en sufrir atresia (Driancourt et al., 1991; Tajima et al., 2002; Yang y Rajamahendran, 2000). Parece ser que el progreso espacial o temporal de la atresia folicular no se produce de forma lineal, sino que es necesario que se alcance un nivel umbral de células apoptóticas en el folículo para que el ovocito se vea afectado (Zeuner et al., 2003).

Se ha observado que los folículos subordinados en estadíos tempranos de atresia contienen ovocitos con capacidad para dar lugar a embriones viables (Vassena et al., 2003), posiblemente debido a la existencia de señales de maduración similares en el folículo dominante maduro y los folículos en etapas tempranas de atresia (Sirard et al., 1999). Se ha sugerido que cambios apoptóticos moderados en el folículo podrían inducir cambios similares a la premaduración que tiene lugar en los ovocitos de folículos pre-ovulatorios, como la expansión de las células del cumulus (de Loos et al., 1991) o la reanudación de la meiosis (Assey et al., 1994). La degeneración de los folículos conduce a una disminución de los niveles de 17ß-estradiol y testosterona, y un aumento de los niveles de progesterona (Kruip y Dieleman, 1985, 1989), que mimetizan los cambios que suceden después del pico LH, influenciando la capacidad para el desarrollo del ovocito. En cambio, los ovocitos de folículos sanos están bloqueados para reanudar la meiosis y completar la maduración citoplasmática (Hendriksen et al., 2000). Por otro lado, los folículos en estado avanzado de atresia contienen ovocitos con una capacidad muy reducida para desarrollarse hasta el estadío de blastocisto (Blondin y Sirard, 1995; de Wit et al., 2000; Zeuner et al., 2003). Teniendo en cuenta que más del 50 % de los folículos presentes en el ovario en un momento determinado son atrésicos (Kruip y Dieleman, 1982), y que de la

mayoría de estos folículos se obtienen ovocitos para ser utilizados en programas de producción *in vitro* de embriones, estos resultados indican que estos folículos, aunque estén atrésicos, podrían contener ovocitos con competencia para el desarrollo.

### *2.3.3.2. Apoptosis en el embrión pre-implantacional*

La apoptosis ha sido observada en embriones pre-implantacionales de la mayoría de mamíferos (revisado por Hardy, 1999), y tiene por finalidad la eliminación de las células sobrantes o anormales (Hardy et al., 2003). En general se considera que el embrión necesita niveles moderados de apoptosis para tener un desarrollo adecuado, pero niveles altos de apoptosis son perjudiciales para su desarrollo. Se ha visto que un porcentaje de fragmentación embrionaria del 10-15 % no afecta al desarrollo embrionario (Alikani et al., 2000; Hardy et al., 2003), pero un índice mayor resulta en la incapacidad del embrión para llegar al estadío de blastocisto, probablemente debido a una pérdida de interacción célula-célula que interfiere en la compactación, cavitación y formación del blastocisto (Alikani et al., 1999; Van Blerkom et al., 2001). En general, la apoptosis no se observa en embriones con desarrollo normal antes de que se produzca la activación del genoma embrionario, excepto cuando se induce por un factor externo (revisado por Fabian et al., 2005). La aparición de células apoptóticas en embriones normales se da después de la activación del genoma embrionario, aunque el momento exacto depende de la especie: en ratón y cerdo la apoptosis aparece en estadío de blastocisto, en bovino en estadío de 6-8 células, y en humano la apoptosis aparece en el embrión cuando se da la compactación (revisado por Fabian et al., 2005). Parece ser que la falta de apoptosis antes de la activación del genoma embrionario responde a la falta de transcripción, ya que el embrión utiliza el pool de ARNm materno almacenado para mantener su actividad celular, con lo que la presión selectiva para mantener el ADN intacto o eliminar el dañado desaparece. Por otro lado, en el embrión temprano no hay puntos de control (*checkpoints*) a lo

largo del ciclo celular que normalmente monitorizan la integridad del genoma, y la aparición de los puntos de control coincide con la aparición de la muerte celular (revisado por Greenwood y Gautier, 2005).

La apoptosis se ha observado tanto en embriones producidos *in vivo* como *in vitro* (Hardy, 1999; Hardy y Spanos, 2002; Pomar et al., 2005; revisado por Gjorret et al., 2003) , aunque la incidencia aumenta en los embriones producidos *in vitro* (Pomar et al., 2005; revisado por Fabian et al., 2005). Este hecho podría estar relacionado con una menor calidad de estos embriones (Hardy et al., 1989).

Los factores que pueden desencadenar apoptosis en el embrión son variados. En numerosas especies se ha observado que la apoptosis en el embrión puede estar causada por unas condiciones de cultivo *in vitro* subóptimas (Brison y Schultz, 1998; Gjorret et al., 2003; Hardy, 1999; Kidson et al., 2004; Makarevich y Markkula, 2002; Morgan et al., 1995; Moussa et al., 2004; Pampfer et al., 2001; Rizos et al., 2002). También es posible que sea la continuación de un proceso iniciado en los gametos, en particular en el ovocito (Hardy et al., 2001; Jurisicova et al., 1998; Lévy, 2005), ya que se ha descrito la presencia de moléculas pro-apoptóticas en el pool de ARNm almacenado en el ovocito humano y en el murino (Exley et al., 1999; Jurisicova et al., 1998; Metcalfe et al., 2004), así como una influencia del genotipo materno en la fragmentación celular del futuro embrión (Han et al., 2005). No obstante, la incidencia de apoptosis en embriones también puede ser consecuencia de la fecundación de ovocitos por espermatozoides con el ADN dañado (Fatehi et al., 2006) o apoptóticos (Host et al., 2000). Otros factores desencadenantes de apoptosis en el embrión son la presencia de anomalías cromosómicas (Hardy, 1999; Munne et al., 1993), incapacidad para que el embrión pueda mantener el desarrollo (Handyside y Hunter, 1986), o la exposición a factores nocivos (Paula-Lopes y Hansen, 2002; Yang et al., 1998).

## 2.3. PRODUCCION *IN VITRO* (PIV) DE EMBRIONES

### 2.3.1. Animales adultos vs. Prepúberes

Diversos laboratorios utilizan hembras prepúberes como donantes de ovocitos debido a las ventajas que presentan respecto a las hembras adultas. Así, se puede reducir el intervalo generacional, de modo que se acelera la propagación genética de los animales de alto valor, y el número de ovocitos que se consiguen es mucho mayor (Koeman et al., 2003). Además, se ha demostrado que los ovocitos de hembras prepúberes pueden ser madurados y fecundados *in vitro* con éxito (Kajihara et al., 1991; Martino et al., 1994a), utilizarse para producir embriones *in vitro* (caprino: Izquierdo et al., 2002; Izquierdo et al., 1999; Mogas et al., 1997b; bovino: Armstrong et al., 1992; Armstrong et al., 1994; Damiani et al., 1996; Palma et al., 2001; ovino: Ledda et al., 1997; Ledda et al., 1999) y obtener gestaciones y nacimientos (Armstrong et al., 1992).

Sin embargo, la eficiencia en la PIV se ve reducida al utilizar animales prepúberes en comparación con el uso de adultas (revisado por Armstrong, 2001). Esta baja eficiencia se ha relacionado con un menor diámetro ovocitario, menor tasa metabólica, menor síntesis proteica (Gandolfi et al., 1998), menor tasa de maduración meiótica y de capacidad para desarrollarse hasta blastocisto (Kochhar et al., 2002; Marchal et al., 2001) y mayor tasa de polispermia en los ovocitos procedentes de hembras prepúberes (Marchal et al., 2001), todo ello debido a deficiencias en la maduración citoplasmática (Damiani et al., 1996; Salamone et al., 2001). Una de las posibles causas de la deficiente maduración podría ser el almacenaje subóptimo de ARNm (Fulka et al., 1998; Hyttel et al., 1997), ya que se ha visto que hay menor cantidad de ARNm almacenado en ovocitos prepúberes inmaduros (Leoni et al., 2004), o una menor actividad del MPF en ovocitos de hembras prepúberes en comparación con adultas (Salamone et al., 2001) que daría lugar a una menor formación del pronúcleo masculino tras la fecundación (Naito et al., 1992) y, en consecuencia, un menor desarrollo embrionario.

Aunque las deficiencias citoplasmáticas en los ovocitos de las hembras prepúberes han sido ampliamente demostradas, hay que destacar que también han surgido estudios que no han detectado diferencias entre los ovocitos procedentes de animales prepúberes y adultos en cuanto a eficiencia en la maduración meiótica (Armstrong et al., 1992; Koeman et al., 2003; Martino et al., 1995) fecundación (Koeman et al., 2003; Mogas et al., 1997a) y desarrollo embrionario hasta blastocisto (caprino: Izquierdo et al., 2002; Koeman et al., 2003; Mogas et al., 1997a; bovino: Armstrong et al., 1994; ovino: Ledda et al., 1997).

## 2.3.2 Obtención de los ovocitos

### 2.3.2.1. Obtención de los ovarios

La mayor parte de los ovocitos que se utilizan para MIV, FIV y CIV provienen de ovarios de hembras sacrificadas en matadero, ya que permite obtener un gran número de ovarios a coste muy bajo. Sin embargo, el estado fisiológico de las hembras sacrificadas donantes de ovocitos es muy variable, hecho que se ve reflejado en la heterogeneidad de la población ovocitaria que se recupera de esos ovarios.

Existen otras técnicas que permiten la obtención de ovocitos de hembras vivas, entre las que cabe destacar la punción folicular por vía vaginal (OPU) o guiada por laparoscopia (LOPU). Esta técnica tiene la ventaja que permite conocer el estado fisiológico de la hembra, de forma que no afectará a la recuperación de los ovocitos (Galli et al., 2001). Por otro lado, las hembras también pueden ser estimuladas hormonalmente para recuperar un mayor número de ovocitos. Sin embargo, el coste de la OPU es mucho más elevado que la obtención de ovarios de hembras sacrificadas en matadero, y el número de ovocitos obtenido es muy inferior.

2.3.2.2. Métodos de obtención de ovocitos

Son varios los métodos que se pueden utilizar para obtener los ovocitos que serán utilizados para la producción *in vitro* de embriones. A continuación describiré sólo aquellos métodos que se utilizan a partir de ovarios de hembras sacrificadas:

a) **Disección folicular**: consiste en separar el folículo intacto del ovario mediante el uso de un bisturí, y una vez aislado se recupera el complejo cumulus-ovocito (COC). Es una técnica muy laboriosa y que requiere mucho tiempo; sin embargo, permite recuperar gran número de COCs y mantener su integridad (Nowshari, 2005), así como conocer perfectamente las características de los folículos de los que proceden los COCs obtenidos.

b) **Aspiración folicular**: consiste en aspirar el contenido folicular con una aguja conectada a una jeringa, donde se va recogiendo el líquido folicular y los COCs que contienen. Es una técnica muy utilizada, ya que permite recuperar un gran número de COCs en relativamente poco tiempo. Sin embargo, presenta algunas desventajas: algunos ovocitos pierden las células del cumulus durante el proceso, la cantidad de COCs recuperados es menor que con otras técnicas (Nowshari, 2005), y a veces se aspira el contenido de los folículos adyacentes sin pretenderlo. Esta técnica, además, requiere la presencia de folículos grandes para ser aspirados, lo que limita su uso a los ovarios procedentes de animales adultos.

c) ***Slicing*** (Martino et al., 1994b): consiste en realizar pequeños cortes en la superficie del ovario con la ayuda de un bisturí, de modo que se rompe la pared folicular y el COC se libera al medio de cultivo donde están sumergidos los ovarios. Es una técnica que permite recuperar gran cantidad de COCs por ovario. Sin embargo la población ovocitaria que se recupera es muy heterogénea, encontrando ovocitos con diferentes grados de desarrollo y atresia, de modo que tras la recuperación debe hacerse una estricta selección de los COCs que se utilizarán para la MIV. A pesar de sus desventajas, es la

técnica más utilizada para recuperar ovocitos de ovarios de hembras prepúberes, ya que suelen carecer de folículos de tamaño considerable.

## 2.3.3. Selección de los ovocitos

La heterogeneidad de la población ovocitaria obtenida a partir de hembras sacrificadas hace necesario realizar una cuidadosa selección de los ovocitos con el objetivo de utilizar sólo aquéllos que han adquirido la competencia para dar lugar a un embrión viable. Se han realizado numerosos estudios con el objetivo de encontrar marcadores que pudieran ser indicadores de la competencia para el desarrollo de los ovocitos. Es necesario que estos marcadores permitan una selección rápida y no perjudicial de los ovocitos, con lo que la mayoría de estudios se han centrado en parámetros morfológicos que pudieran ser evaluados visualmente.

### 2.3.3.1. Métodos no invasivos

#### *2.3.3.1.a. Tamaño folicular*

La selección de los ovocitos dependiendo del tamaño folicular es uno de los métodos más utilizados, sobre todo cuando se realiza la disección o aspiración de los folículos. La relación entre el tamaño folicular y la competencia para el desarrollo de los ovocitos obtenidos se ha observado en numerosas especies:

- En **bovino**, Lonergan et al. (1994) observaron que el porcentaje de blastocistos obtenidos a partir de ovocitos liberados de folículos > 6 mm de diámetro era mayor que en folículos de diámetro 2-6 mm. Blondin y Sirard (1995) determinaron que los folículos de vacas que medían menos de 3 mm contenían ovocitos que no eran capaces de desarrollarse hasta el estadío de blastocisto, mientras que Lequarre et al. (2005) observaron diferente tasa de desarrollo hasta blastocisto en

ovocitos procedentes de folículos > 6 mm en comparación con los de folículos < 4 mm, aunque el número de células de los blastocistos obtenidos no dependía del tamaño folicular. En terneras el resultado también ha sido similar, y se han obtenido mayores porcentajes de blastocistos a partir de los ovocitos recuperados de folículos de diámetro superior a 8 mm (Kauffold et al., 2005).

- En **búfalo** también se ha observado que los ovocitos obtenidos de los folículos de mayor diámetro (> 8 mm) dan lugar a un mayor porcentaje de blastocistos comparados con los ovocitos obtenidos de folículos medios (3-8 mm) o pequeños (< 3 mm) (Raghu et al., 2002).

- En **cabras adultas**, Crozet et al. (1995) observaron que se obtenía mayor porcentaje de blastocistos con ovocitos obtenidos de folículos > 5 mm en comparación con los que procedían de folículos medianos (3.1-5 mm) y pequeños (2-3 mm).

- En **ovejas**, Ledda et al. (1999) también observaron que la progresión meiótica de los ovocitos estaba relacionada con el tamaño de los folículos, tanto en animales adultos como en prepúberes.

La diferencia en la competencia ovocitaria según el tamaño folicular se debe posiblemente a que en los folículos de mayor diámetro existen factores que favorecen la capacidad para el desarrollo del ovocito que contienen, ya que Algriany et al. (2004) observaron que al añadir al medio de maduración líquido folicular de folículos grandes el desarrollo embrionario mejoraba en comparación con el líquido folicular que provenía de folículos pequeños.

*2.3.3.1.b. Diámetro ovocitario*

En numerosas especies se ha observado que el diámetro de un ovocito está relacionado con el tamaño del folículo que lo contiene (vaca: Arlotto et al., 1996; cabra: Crozet et al., 2000; de Smedt et al., 1994; búfalo: Raghu et

al., 2002) como el tamaño folicular, a la vez, se correlaciona con la competencia meiótica del ovocito (ratón: Sorensen y Wassarman, 1976; rata: Bar-Ami y Tsafriri, 1981; cerdo: Motlik et al., 1984**;** oveja**:** Moor y Gandolfi, 1987; vaca: Furher et al., 1989; cabra: de Smedt et al., 1994; Martino et al., 1994a; mono: Schramm et al., 1993) y su capacidad para mantener el desarrollo embrionario (cabra: Crozet et al., 1995; vaca: de Wit y Kruip, 2001; Kauffold et al., 2005; Lequarre et al., 2005; búfalo: Raghu et al., 2002).

En cabras adultas, de Smedt et al. (1994) clasificaron los ovocitos según su diámetro en 4 grupos: 1) < 110 µm, ovocitos que provenían de folículos de 0.5-0.8 mm de diámetro, considerados incompetentes; 2) 110-125 µm, los ovocitos procedentes de folículos de diámetro 1-1.8 mm, considerados parcialmente competentes; 3) y ovocitos de diámetro 125-135 y 4) > 135 µm, procedentes de folículos mayores de 3 mm de diámetro, considerados meióticamente competentes.

En todos los trabajos realizados en esta tesis se han seleccionado los ovocitos según su diámetro, siguiendo la clasificación realizada por de Smedt et al. (1994) por 2 motivos: 1) la técnica utilizada para la recuperación de los ovocitos ha sido el slicing, técnica incompatible con la selección por tamaño folicular, y 2) creemos que cuando se trabaja con animales prepúberes el diámetro es el método de selección mas fiable en comparación con la selección según el tamaño folicular, ya que se ha observado que folículos del mismo tamaño contienen ovocitos de menor diámetro en hembras prepúberes que en adultas (bovino: Gandolfi et al., 1998; ovino: Ledda et al., 1999).

*2.3.3.1.c. Morfología del COC*

La posibilidad de seleccionar los ovocitos según su morfología fue investigada por primera vez en el bovino (Leibfried y First, 1979). Numerosos estudios han indicado la utilidad de la morfología del COC, basándose en la compactación y en el número de capas de células del cumulus, así como en la

homogeneidad del citoplasma del ovocito, para la selección de ovocitos inmaduros con mayor capacidad para madurar, ser fecundados y desarrollarse *in vitro* (Brackett y Zuelke, 1993; Madison et al., 1992). Además, Salamone et al. (1999) observaron que la morfología del COC y la competencia del ovocito variaban durante las fases de crecimiento y degeneración de los folículos subordinados, lo que refuerza su posible uso como indicador de competencia.

Por otro lado, la morfología del COC también se ha relacionado con el grado de atresia del folículo del cual proviene (Blondin y Sirard, 1995; de Wit et al., 2000). En general, se acepta que los COCs que presentan varias capas de células del cumulus compactas y el citoplasma del ovocito homogéneo provienen de folículos no atrésicos (de Wit et al., 2000) y son los que poseen mayor competencia para el desarrollo (Corn et al., 2005; Lee et al., 2001; Yuan et al., 2005; Zeuner et al., 2003). Esta morfología estaría relacionada con una mejor redistribución de las mitocondrias después de la MIV y un mayor contenido de ATP (Stojkovic et al., 2001). Sin embargo, otros estudios han observado una mayor capacidad para el desarrollo embrionario en aquellos COCs que presentaban signos leves de atresia evaluados morfológicamente, como el citoplasma el ovocito granulado y/o con las capas de célula del cumulus expandidas en diferentes grados (Blondin y Sirard, 1995; Boni et al., 2002; de Wit et al., 2000).

*2.3.3.1.d. Otros métodos no invasivos*

a) **Morfología del ovario**: Gandolfi et al. (1997) observaron que, en bovino, los COCs recuperados de ovarios con un folículo mayor de 10 mm de diámetro o más de 10 folículos de entre 2-5 mm de diámetro daban lugar a un mayor porcentaje de blastocistos que aquéllos obtenidos de ovarios con menos de 10 folículos de entre 2-5 mm diámetro.

b) **Concentración de diferentes componentes en el líquido folicular**: La concentración de estradiol (Anifandis et al., 2005; Wunder et al., 2005), leptina (Anifandis et al., 2005), inhibina B (Chang et al., 2002) y mio-inositol (Chiu et al., 2002) en el líquido folicular se ha relacionado con una mayor capacidad ovocitaria para dar lugar a embriones de mejor calidad, mayor tasa de implantación y gestaciones.

2.3.3.2. Métodos invasivos

Todas las técnicas invasivas se basan en métodos moleculares, como la PCR, el radiomarcaje o la tinción con fluorocromos, que impiden que el ovocito pueda seguir con su desarrollo. Por este motivo, es necesario relacionar características moleculares del ovocito con características que puedan ser evaluadas visualmente, como los métodos no invasivos mencionados anteriormente. Algunas de las características moleculares que se han relacionado con la competencia ovocitaria se describen a continuación.

*2.3.3.2.a. Contenido mitocondrial del ovocito*

Santos et al. (2006) observaron que el contenido mitocondrial de los ovocitos, tras haber realizado un protocolo de FIV o ICSI *(Intracytoplasmic sperm injection)*, era superior en los ovocitos fecundados que en los que no presentaban extrusión del segundo corpúsculo polar o formación de los pronúcleos (considerados no fecundados), con lo que podría ser un buen marcador para predecir el éxito de la fecundación *in vitro*.

*2.3.3.2.b. Abundancia de transcritos específicos*

Se ha observado una relación de la morfología del ovocito, y por tanto de su competencia meiótica, con la abundancia relativa de determinados transcritos en el ovocito (De Sousa et al., 1998). En embriones considerados

de buena calidad se ha observado una mayor transcripción de genes relacionados con el estrés, con el metabolismo (Gutiérrez-Adán et al., 2004) y con la regulación de la expresión génica (Dode et al., 2006).

*2.3.3.2.c. Grado de apoptosis*

Las técnicas invasivas más utilizadas hasta ahora para evaluar el grado de apoptosis, tanto en el ovocito y en las células del cumulus que lo rodean como en los embriones resultantes, han sido la técnica de TUNEL (Corn et al., 2005; Hao et al., 2004; Lee et al., 2001; Van Blerkom y Davis, 1998; Vandaele et al., 2006; Warzych et al., 2006; Wu et al., 2000; Yuan et al., 2005; Zeuner et al., 2003), la tinción con Annexin-V (Giampietro et al., 2006; Tseng et al., 2006; Van Blerkom and Davis, 1998), la expresión relativa de Bax y Bcl-2 (Yang y Rajamahendran, 2002), o la actividad de caspasas (Bilby et al., 2006; Bosco et al., 2005; Ortiz et al., 2006).

Generalmente, se ha observado que la capacidad para el desarrollo embrionario se incrementa en los complejos cumulus-ovocito que presentan menor incidencia de apoptosis, evaluada mediante técnicas invasivas (Corn et al., 2005; Lee et al., 2001; Yang y Rajamahendran, 2002; Yuan et al., 2005; Zeuner et al., 2003). Curiosamente, estos resultados contradicen los estudios que relacionaban la presencia de signos leves de atresia, evaluados según la morfología de los COCs, con su mayor capacidad para desarrollarse hasta blastocisto (Bilodeau-Goeseels y Panich, 2002; Blondin y Sirard, 1995).

# 3. OBJETIVOS

El objetivo principal de esta tesis es la búsqueda de marcadores moleculares que nos ayuden a determinar la calidad del ovocito para poder predecir su capacidad de desarrollarse hasta blastocisto después de un proceso de maduración y fecundación *in vitro*. Para su consecución, nos planteamos los siguientes objetivos:

- Estudiar la relación entre el diámetro del ovocito y su competencia para dar lugar a un embrión viable y así poder utilizar este parámetro como indicador no invasivo de competencia ovocitaria.

- Analizar la expresión de las dos subunidades del MPF, así como su actividad y la acumulación de RNA y de proteína totales en ovocitos de diferente diámetro, con la finalidad de valorar su posible implicación en la adquisición de competencia del ovocito para el desarrollo embrionario.

- Valorar el efecto de la incidencia de la apoptosis, tanto en ovocitos como en células del cumulus, en la adquisición de competencia ovocitaria para el desarrollo embrionario, así como su efecto en los blastocistos resultantes.

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# Effect of oocyte diameter on meiotic competence, embryo development, p34 (cdc2) expression and MPF activity in prepubertal goat oocytes

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**Abstract**

The aim of this study was to analyze the relationship between oocyte diameter, meiotic and embryo developmental competence and the expression of the catalytic subunit of MPF, the $p34^{cdc2}$, at mRNA, RNA and protein level, as well as its kinase activity, in prepubertal (1–2 months old) goat oocytes. MPF is the main meiotic regulator and a possible regulator of cytoplasmic maturation; therefore, it could be a key factor in understanding the differences between competent and incompetent oocytes. Oocytes were classified according to oocyte diameter in four categories: <110, 110–125, 125–135 and >135 μm and matured, fertilized and cultured in vitro. The $p34^{cdc2}$ was analyzed in oocytes at the time of collection (0 h) and after 27 h of IVM (27 h) in each of the oocyte diameter categories. The oocyte diameter was positively related to the percentage of oocytes at MII after IVM (0, 20.7, 58 and 78%, respectively) and the percentage of blastocysts obtained at 8 days postinsemination (0, 0, 1.95 and 12.5%, respectively). The expression of RNA and mRNA $p34^{cdc2}$ did not vary between oocyte diameters at 0 and 27 h. Protein expression of $p34^{cdc2}$ increased in each oocyte category after 27 h of maturation. MPF activity among diameter groups did not vary at 0 h but after IVM there was a clear and statistically significant increase of MPF activity in the biggest oocytes.


*Keywords:* Goat; Oocyte diameter; IVF; MPF

## 1. Introduction

Mammalian oocytes are arrested at the dictyate stage of meiosis, also known as germinal vesicle (GV) stage, during their growth. Fully grown oocytes are able to resume meiosis in vivo after the LH surge, or spontaneously after their release from the follicle and subsequent in vitro culture [1]. In contrast, growing oocytes are not able to resume or complete meiosis. Oocyte competence is acquired during the growth phase, when the synthesis and storage of proteins and ribosomal and heterogeneous RNA take place [2] and implies its ability to complete not only nuclear maturation, but also cytoplasmic changes needed to maintain embryo development. These cytoplasmic changes include: (a) protein and RNA storage, (b) development of calcium regulatory mechanisms, (c) changes in the activity of MPF and MAPK, (d) redistribution of cellular organelles, etc. The regulation of cytoplasmic maturation is not as well known as nuclear maturation regulation, however Naito et al. [3] suggested that MPF plays an important part in this

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process. MPF is a heterodimer composed of a $p34^{cdc2}$ catalytic subunit, with serine-threonine kinase activity, and a cyclin B1 regulatory subunit [4]. While the association of these two subunits is a requirement for the activation of protein kinase activity, the phosphorylation of $p34^{cdc2}$ on threonine 161 by the protein kinase CAK (Cdc2 activating kinase) and dephosphorylation on threonine 14 and tyrosine 15 by the Cdc25 phosphatase is also necessary [5]. MPF activity has been described in many mammalian oocytes: it appears just before germinal vesicle breakdown (GVBD) and increases until metaphase I (MI), its activity decreases in anaphase–telophase and increases again, reaching its maximum level at metaphase II (mouse: [6–8]; pig: [9]; rabbit: [10]; cattle: [11]; goat: [12]; sheep: [13]). MPF activity has been shown to be lower in prepubertal than in adult female oocytes (cow: [14]; sheep: [13]).

Several studies of prepubertal animals have reported low embryo development (revised by Armstrong [15]). In adult females, the diameter of follicles has been positively related to oocyte diameter (cattle: [16]; buffalo: [17]) and to oocyte developmental competence (cattle: [18]; goat: [19]; pig: [20]). In adult goats, Crozet et al. [19] found a direct and positive relationship between follicular diameter and embryo development, concluding that follicles larger than 5 mm contain oocytes which are more competent to develop up to blastocyst stage. Prepubertal goat ovaries contain a great number of small follicles, most of them between 2.5 and 3 mm in diameter [21]. This means that follicles larger than 5 mm in diameter are practically non-existent in these ovaries. When the slicing technique was used to liberate oocytes from ovaries, the number of selected oocytes per ovary was 6.05, the rest of the oocytes were denuded, degenerated or oocytes with partially or totally expanded cumulus [22]. The blastocyst yield in these prepubertal goat oocytes is lower [23] than oocytes from adult goats [19]. In both adult [24] and prepubertal [21] goat oocytes meiotic oocyte competence has been classified in terms of the oocyte diameter as follows: $<110$ μm, corresponding to incompetent oocytes, 110–125 μm, corresponding to partially competent oocytes, 125–135 and $>135$ μm, corresponding to competent oocytes. No differences in meiotic competence were found between adult and prepubertal oocyte diameters [21]. However, embryonic development related to oocyte diameter has not been studied in prepubertal goat oocytes.

The objective of this study was to find a relationship between oocyte diameter, embryo development, and the expression of the catalytic subunit of MPF, the $p34^{cdc2}$, at mRNA, RNA and protein level, as well as its kinase activity, in prepubertal goat oocytes.

## 2. Material and methods

### 2.1. Oocyte collection and in vitro maturation

Ovaries from prepubertal goats, approximately 2 months old, were recovered from a local slaughterhouse and transported within 2 h to the laboratory at 38.5 °C in PBS (Dulbecco's phosphate-buffered saline, Sigma Chemical Co., St. Louis, MO, USA) containing 50 μg/ml gentamycin (Sigma, USA). Ovaries were washed three times in PBS containing gentamycin, and oocytes were recovered by slicing in a 60 mm culture dish containing TCM199 (Sigma, USA) supplemented with 2.2 mg/ml $NaHCO_3$, 2% (v/v) steer serum (Donor Bovine Serum®, Canada) and 50 μg/ml gentamycin. Only oocytes with two or more layers of cumulus cells and homogeneous cytoplasm were used for IVM.

A sample of oocytes recovered was denuded in PBS + 300 μg/ml hyaluronidase (Sigma, USA), measured at 96× magnification excluding the zona pellucida and classified by diameters in four classes: $<110$, 110–25, 125–135 and $>135$ μm. These oocytes were used to evaluate the nuclear stage by lacmoid staining at collection time.

The rest of oocytes were matured in TCM199 medium (Sigma, USA) supplemented with 275 μg/ml sodium pyruvate (Sigma, USA), 146 μg/ml L-glutamine (Sigma, USA), 10% (v/v) steer serum, 10 μg/ml o-LH (Sigma, USA), 10 μg/ml o-FSH (Sigma, USA), 1 μg/ml 17-β estradiol (Sigma, USA), 400 μM cysteamine (Sigma, USA) and 50 μg/ml gentamycin (Sigma, USA). Groups of 20–25 cumulus enclosed oocytes (COCs) were transferred to 100 μl microdrops of maturation medium and incubated for 27 h at 38.5 °C in a humidified air atmosphere with 5% $CO_2$ under mineral oil (Sigma, USA).

After IVM, a sample of oocytes were denuded in sodium citrate 2.3%, classified by diameters as described before and used to evaluate the nuclear stage by lacmoid staining.

After and before maturation, a sample of oocytes were denuded, measured as described above, washed three times in PBS and frozen in groups of 10 in $N_2$ liquid and stored at −80 °C until the extraction of RNA.

### 2.2. Sperm capacitation and in vitro fertilization

Oocytes were fertilized with fresh semen collected from two goats of proven fertility. Ejaculates were collected with artificial vagina, and transported to the laboratory at 38.5 °C within 30 min. Massal motility of the ejaculates was evaluated. Motile sperm fraction was

obtained using the swim-up method [25] whereby 70 μl semen were layered below 2 ml of defined medium ([26], modified by Younis et al. [27]), referred to here as mDM, and it was incubated for 45–60 min in a humidified air atmosphere with 5% $CO_2$ at 38.5 °C. After incubation, 600 μl of the supernatant of each tube was recovered and centrifuged at 500 rpm for 10 min. After discarding the supernatant, the pellet was resuspended in proportion 1:1 with mDM containing heparin (Sigma, USA) and ionomycin (Sigma, USA) (final concentration: 10 μg/ml heparin and 200 nM of ionomycin), and incubated for 15 min in a humidified air atmosphere with 5% $CO_2$ at 38.5 °C [28]. Sperm concentration was determined using a Neubauer chamber cell.

After maturation, we transferred groups of 20–25 oocytes to 100 μl microdrops of modified Tyrode's medium (TALP) as described by Parrish et al. [25], supplemented with 1 μg/ml hypotaurine (Sigma, USA), under mineral oil. Oocytes were co-cultured with capacitated spermatozoa in a final concentration in the microdrop of $4 \times 10^6$ cells/ml.

A sample of oocytes were recovered after 17 h of IVF, denuded with sodium citrate 2.3% and classified by diameters to evaluate fertilization rate.

### 2.3. Embryo in vitro culture

At 24 h after insemination, zygotes were washed to remove both sperm cells and remnant cumulus cells, and they were classified by diameters as described above. Groups of 18–25 embryos of the same diameter were placed in 25 μl microdrops of culture medium (SOF, synthetic oviductal fluid [29]; modified by Takahashi and First [30]) supplemented with aminoacids and BSA [31] in 35 mm culture dishes under mineral oil, and they were cultured in a humidified atmosphere with 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5 °C for 7 days. At 48 h after insemination, we added 0.1 μl foetal bovine serum (FBS, Life Technologies) per embryo. At the end of the culture period, total cell number of embryos was evaluated by Hoescht 33342 staining (Sigma, USA) under a fluorescence microscope, and the percentage of total embryos (embryos with two blastomeres or more), morulae (embryos with 16 or more cells and without blastocoele) and blastocysts (embryos with 60 or more cells and with blatocoele formation) was recorded.

### 2.4. Evaluation of nuclear stage

To evaluate the nuclear stage of oocytes at collection time, after IVM and after IVF, oocytes were fixed in ethanol: acid acetic (3:1, v/v) for 24 h at 4 °C. After that time, oocytes were stained with 1% lacmoid (Sigma, USA) in 45% acetic acid.

The classification of oocytes at collection time and after IVM was the following: germinal vesicle (GV), when the nuclear membrane and the nucleolus were visible and chromatin was not condensed; germinal vesicle breakdown (GVBD), when the nuclear membrane and the nucleolus were not visible, and chromosomes had begun to condense; metaphase I (MI), when the chromosomes were condensed and arranged in the metaphase spindle; anaphase–telophase (A–T), when homologous chromosomes begin to separate; and metaphase II (MII), when the metaphase plate and the first polar body were observed.

After IVF, oocytes with a sperm tail in the cytoplasm were considered fertilized and they were classified into one of the three groups: asynchrony, when a condensed sperm head and the female pronucleus were visible in the cytoplasm; 2PN (normal fertilization): the two pronuclei (male and female) and one sperm tail were visible in the cytoplasm; and polyspermy, when there were two or more sperm tails with condensed or descondensed sperm heads in the cytoplasm. Oocytes without pronuclei and sperm tails in the cytoplasm were classified as no fertilized.

### 2.5. RNA extraction

Total RNA was extracted from groups of 10 oocytes using 100 μl of TriReagent® (T-9424, Sigma, USA). We added 20 μg of glycogen (Roche, Germany) to the 100 μl as a carrier for the RNA precipitation, and 1 pg of rabbit globin mRNA per oocyte (Sigma, USA), that was used as an extrinsic control of the whole process. After adding 20 μl of chloroform, we centrifuged the sample at 12 000 × *g* for 20 min at 4 °C. The aqueous phase was recovered and the RNA was precipitated with 60 μl of isopropanol at −20 °C overnight, while the resulting organic phase was frozen at −80 °C until its use in protein extraction. The next day, after centrifugation at 12 000 × *g* at 4 °C for 20 min, the supernatant was discarded and the pellet was washed sequentially with 70% ethanol and 100% ethanol. For the washing steps, the sample was centrifuged at 12 000 × *g* at 4 °C for 15 min. The pellet was dried and resuspended in 4 μl of $H_2O$ DEPC.

The RNA extracted was incubated for 30 min at 37 °C with 1U/μl DNase (Invitrogen Corporation, USA), and stored at −80 °C.

### 2.6. Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA corresponding to 10 oocytes was reverse-transcribed with *ThermoScript*™ *RT-PCR System* (Invitrogen Corporation, USA), following the manufacturer's instruction, in a final volume of 20 μl. The RT was performed using oligodT and random primers. Four microliters of these reactions were used to amplify $p34^{cdc2}$.

To perform $p34^{cdc2}$ amplification, cDNA was incubated with 1× PCR buffer, 0.5 μM of each primer, 2 mM of $MgCl_2$, 200 μm of each dNTP and 0.1 U/μl of Taq polymerase GoldStar (Eurogentec, Belgium) in a final volume of reaction of 25 μl for 36 cycles (94 °C 1 min, 56 °C 1 min, 72 °C 1 min) in a thermocycler 9700 (Applied BioSystems). The $p34^{cdc2}$ sense and antisense primers were the following: 5′-GAATTAGCAACGAAGAAACC and 5′-GTACAATATATCTGCTCTTGAC [32].

To perform globin amplification, cDNA was incubated with 1× PCR buffer, 0.5 μm of each primer, 1.5 mM of $MgCl_2$, 200 μm of each dNTP and 0.1 U/μl of Taq polymerase GoldStar in a final volume of 25 μl for 30 cycles. Globin PCR conditions were determined by the amplification conditions of the gene. The globin sense and antisense primers were the following: 5′-GCAGCCACGGTGGCGAGTAT and 5′-GTGGGACAGGAGCTTGAAAT [33].

At the same time, a PCR without cDNA and a PCR of the RT product performed without RNA were used as negative controls. In no case, an amplification band was detected.

Amplification bands were visualized in 2% agarose gel stained with ethidium bromide. The weight marker band (Sigma, USA) corresponding to 300 pb was used as a control to avoid light variations in the exposition to UV. Each amplification band was quantified by densitometric analysis using the PC program *Quantity One* (Bio-Rad Laboratories, USA). The results were normalized with the quantification of globin amplification band.

Direct sequencing of the amplification products was performed using an ABI-prism DNA sequencer.

### 2.7. Protein extraction and Western blot

Oocyte protein was extracted using TriReagent® (Sigma, USA) from the organic phase resulting of the RNA extraction. DNA was precipitated with 30 μl of ethanol 100% and centrifuged at 12 000 × *g* at 4 °C for 5 min. The supernatant was recovered and then transferred to a new tube. Proteins were precipitated with 150 μl of isopropanol for 10 min at room temperature, and centrifuged at 12 000 × *g* at 4 °C for 10 min. The supernatant was discarded, and the pellet was washed three times with 200 μl of 0.3 M guanidine hydrochloride/95% ethanol solution. During each wash, samples were stored in wash solution for 20 min at room temperature. Samples were centrifuged at 7500 × *g* for 5 min at 4 °C. After the three washes, we added 1 ml of 100% ethanol and vortered the protein pellet. The sample was left at room temperature for 20 min, and centrifuged at 7500 × *g* for 5 min at 4 °C. We discarded the supernatant, and the protein pellet was dried for 5–10 min. The pellet was dissolved in 20 μl of 1% SDS, and stored at −80 °C until its use for the Western blot.

Polypeptides corresponding to 20 oocytes were separated using a 10% SDS acrylamide gel for 2 h approximately at constant 25 mA. Stained proteins of known molecular weights were run simultaneously as standards. The electrophoretical separated proteins were transferred to a nitrocellulose sheet for 1 h at 100 V. After the transference, sheets were blocked by incubation with TBS 0.05% Tween20 containing 1% milk overnight. We washed with TBS 0.05% Tween20 three times, and nitrocellulose sheet was incubated with the first antibody (1:100, mouse IgG against $p34^{cdc2}$, Santa Cruz Biotechnology) for 1 h. After that incubation, sheets were washed three times with TBS 0.05% Tween20, and incubated with the second antibody (1:2000, anti-mouse IgG antibody) for 30 min. The blot was washed six times with TBS 0.05% Tween20, and its detection was performed using the ECL-Plus Western Blotting Detection Kit (Amersham Biosciences).

### 2.8. In vitro Cdc2 kinase activity assay

Before and after IVM, 20 oocytes of each diameter group were washed three times in PBS, and placed in tubes containing 5 μl of lysis buffer (50 mM Tris–HCl pH 7.5, 0.5 M NaCl, 5 mM EDTA, 0.01% Brij35, 1 mM PMSF, 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β-glycerophosphate, 1 mM Na-orthovanadate). All chemicals were purchased from Sigma. The samples were frozen in liquid nitrogen and sonicated three times at 1 °C for 25 s. Cell extracts were stored at −80 °C until use.

Cdc2 assay was performed using the MESACUP cdc2 kinase assay kit (MBL, Japan) following the manufacturer's protocol. Five microliters of oocyte extract was mixed with 10× cdc2 Reaction Buffer (25 mM Hepes buffer pH 7.5, 10 mM $MgCl_2$) and 10% biotynilated MV

Table 1
Nuclear stage of different sizes of prepubertal goat oocytes at collection time

| Diameter (μm) | No. | GV (%) | GVBD (%) | MI (%) | A–T (%) | MII (%) | DEG (%) |
|---|---|---|---|---|---|---|---|
| <110 | 75 | 62 a (82.66) | 4 a (5.33) | 2 (2.66) | 0 (0) | 0 (0) | 7 a, b (9.33) |
| 110–125 | 105 | 51 b (48.57) | 28 b (26.66) | 5 (4.76) | 0 (0) | 0 (0) | 21 a (20.00) |
| 125–135 | 250 | 34 c (13.60) | 161 c (64.40) | 20 (8.00) | 1 (0.40) | 4 (1.60) | 30 a, b (12.00) |
| >135 | 198 | 3 d (1.51) | 159 d (80.30) | 15 (7.57) | 0 (0) | 4 (2.02) | 17 b (8.58) |

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; A–T: anaphase–telophase; MII: metaphase II; DEG: degenerated oocytes. a, b, c, d: different letters in each column show statistically differences ($P < 0.05$).

Peptide (SLYSSPGGAYC). The phosphorylation reaction was started adding 0.1 mM ATP (Sigma, USA), in a final volume of 50 μl. The mixture was incubated at 30 °C for 30 min. The reaction was finished by adding 200 μl of phosphorylation Stop Reagent (PBS containing 50 mM EGTA). The phosphorylated MV peptide was detected by ELISA at 492 nm.

### 2.9. Statistical analysis

Comparisons between groups were performed using the statistical program Graph-Pad Instat 3.01 for Windows 95 (Graph-Pad software, San Diego, CA, USA). Fisher's exact test was used to determine which diameter groups differed in nuclear stage at collection time, after IVM and after IVF.

PCR semi-quantification results of different diameter groups were compared with a one-way ANOVA test with Tukey's post-test. Comparison between 0 and 27 h IVM of the same diameter group was performed using the *t*-test. For each diameter group, each experiment was performed a minimum of three times.

In every test, values with $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Nuclear stage of oocytes at collection time and after in vitro maturation

Results on the nuclear stages of oocytes at the time of collection are presented in Table 1. The percentages of oocytes arrested at germinal vesicle stage (GV) differed statistically ($P < 0.05$), being higher in the smallest oocytes. As the diameter increased, the percentage of oocytes in GVBD stage also increased ($P < 0.05$).

After 27 h of IVM, the percentage of oocytes that reached MII was significantly higher when oocyte diameter increased, as shown in Table 2.

### 3.2. Nuclear stage of oocytes after in vitro fertilization

Table 3 represents pronuclear stage of prepubertal goat oocytes at 17 h post-fertilization. The percentage of total zygotes (asynchronics, with two pronuclei and polyspermics) was higher when the oocyte diameter increased ($P < 0.05$). In addition, the percentage of zygotes that presented normal fertilization, that is, a male and a female pronuclei, was lower in the smallest oocytes when compared to the other three groups of size ($P < 0.05$).

### 3.3. In vitro embryo development

Embryo development within oocyte size is shown in Table 4. Total number of embryos obtained after the culture period was statistically higher when oocyte diameter increased ($P < 0.05$). Statistical differences in the percentage of 2–7 cells embryos obtained were also observed in oocytes smaller than 125 μm when compared to oocytes larger than 125 μm. There were no statistical differences in the percentage of 8–16 cells and morulae obtained in the four groups of size.

Table 2
Nuclear stage of different sizes of prepuberal goat oocytes at 27 h of IVM

| Diameter (μm) | No. | GV (%) | GVBD (%) | MI (%) | A–T (%) | MII (%) | DEG (%) |
|---|---|---|---|---|---|---|---|
| <110 | 40 | 38 a (95.00) | 1 a, b (25.00) | 0 a (0) | 0 (0) | 0 a (0) | 1 (2.50) |
| 110–125 | 145 | 38 b (26.21) | 11 a (7.58) | 64 b (44.14) | 0 (0) | 30 b (20.69) | 2 (1.38) |
| 125–135 | 327 | 3 c (0.92) | 8 b (2.45) | 124 b (28.03) | 0 (0) | 189 c (57.97) | 3 (0.92) |
| >135 | 91 | 0 c (0) | 1 b (1.10) | 17 c (18.68) | 1 (1.10) | 71 d (78.02) | 1 (1.10) |

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; A–T: anaphase–telophase; MII: metaphase II; DEG: degenerated oocytes. a, b, c, d: different letters in each column show statistically differences ($P < 0.05$).

Table 3
Pronuclear stage of different sizes of prepubertal goat oocytes at 17 h postinsemination

| Diameter (μm) | No. | Total fertil. (%) | Fertilized oocytes | | |
|---|---|---|---|---|---|
| | | | Asynchrony (%) | 2 PN (%) | Polisp. (%) |
| <110 | 24 | 0 a (0) | 0 (0) | 0 a (0) | 0 a (0) |
| 110–125 | 59 | 26 b (44.07) | 2 (3.39) | 17 b (28.81) | 7 a, b (11.86) |
| 125–135 | 93 | 53 b, c (56.99) | 2 (2.15) | 33 b (35.48) | 18 b (19.35) |
| >135 | 82 | 53 c (64.63) | 3 (3.66) | 34 b (41.46) | 16 b (19.51) |

Total fertil.: total fertilized; Asynchrony: oocytes with one female pronucleus and one condensed sperm head; 2PN: zygotes with two pronuclei (normal fertilization); Polisp.: polispermic zygotes. a, b, c: different letters in each column show statistically differences ($P < 0.05$).

Table 4
Embryo development of different sizes of prepubertal goat oocytes at 8 days postinsemination

| Diameter (μm) | No. | Total embryos | Embryos | | | |
|---|---|---|---|---|---|---|
| | | | 2–7 cells | 8–16 cells | Morulae | Blastocysts |
| <110 | 30 | 0 a (0) | 0 a (0) | 0 (0) | 0 (0) | 0 a (0) |
| 110–125 | 74 | 12 b (16.21) | 10 a (13.51) | 2 (2.70) | 0 (0) | 0 a (0) |
| 125–135 | 154 | 61 c (40.13) | 54 b (35.06) | 3 (1.95) | 1 (0.65) | 3 a (1.95) |
| >135 | 72 | 44 d (61.11) | 31 b (43.05) | 2 (2.78) | 2 (2.78) | 9 b (12.50) |

a, b, c, d: different letters in each column show statistically differences ($P < 0.05$).

However, the percentage of blastocysts obtained in the biggest oocytes (12.50%) was statistically higher in respect to the other three groups ($P < 0.05$).

### 3.4. $p34^{cdc2}$ mRNA, RNA and protein expression

The expression of $p34^{cdc2}$ mRNA and total RNA was analyzed by RT-PCR. The primers used in this study were used before by Dedieu et al. [32] in goats. The expected size of the amplified fragment was of 355 pb. The amplification of rabbit globin mRNA by RT-PCR was used as a control of the process, and the fragment obtained had an expected size of 257 pb. We detected $p34^{cdc2}$ mRNA in all diameters of oocytes studied, as shown in Fig. 1.

Results of $p34^{cdc2}$ RNA expression are shown in Fig. 2. We detected $p34^{cdc2}$ RNA in meiotically competent and incompetent oocytes, but we observed statistical differences in the expression of oocytes from 125 to 135 μm at collection time when compared with the other groups ($P < 0.0001$). No differences were detected after IVM.

When studying $p34^{cdc2}$ protein expression, we observed no statistical differences among the four size groups at collection time, although there was a clear tendency to increase the protein expression when the oocyte diameter was higher ($P < 0.10$). However, after IVM, $p34^{cdc2}$ protein expression increased with the oocyte diameter. These results are represented in Fig. 3.

### 3.5. MPF activity

Results of MPF activity in different sizes of prepubertal goat oocytes are presented in Fig. 4. We could see that, although there were no significant differences at collection time among diameter groups,

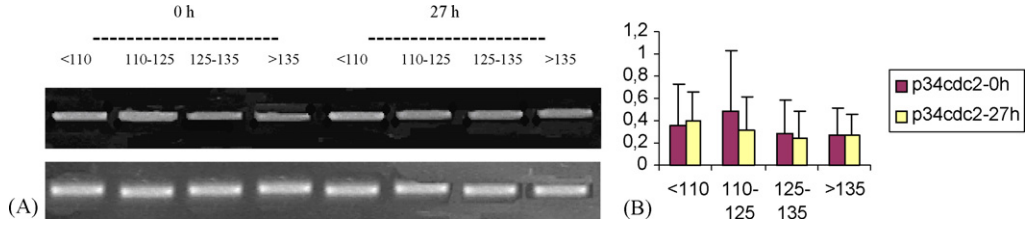


Fig. 1. mRNA $p34^{cdc2}$ expression in different sizes of prepubertal goat oocytes. (A) The $p34^{cdc2}$ RT-PCR product corresponding to 10 oocytes of each diameter group (up) and their corresponding rabbit globin control (down). (B) The histogram below represents the intensity of the bands analyzed by densitometry. Results are expressed as the relationship between the intensity of mRNA $p34^{cdc2}$ expression band and mRNA rabbit globin band.

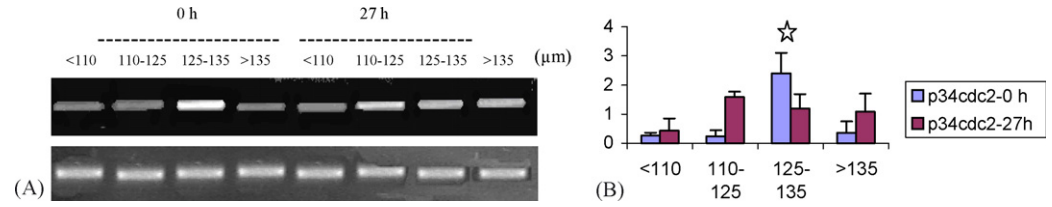


Fig. 2. RNA $p34^{cdc2}$ expression in different sizes of prepubertal goat oocytes. (A) The $p34^{cdc2}$ RT-PCR product corresponding to 10 oocytes of each diameter group (up) and their corresponding rabbit globin control (down). (B) The histogram represents the intensity of the bands analyzed by densitometry. Results are expressed as the relationship between the intensity of RNA $p34^{cdc2}$ expression band and mRNA rabbit globin band. ☆ indicates a significant difference in $p34^{cdc2}$ RNA at collection time ($P < 0.001$).

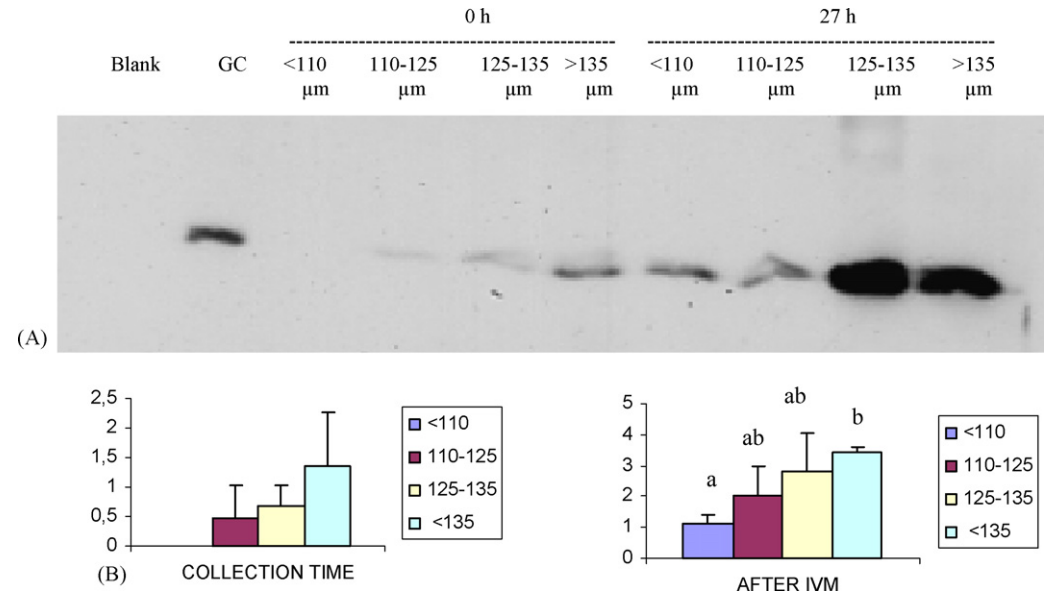


Fig. 3. $p34^{cdc2}$ protein expression in different sizes of prepubertal goat oocytes at collection time (0 h) and after IVM (27 h). (A) Western blot. Each band has a size of 34 kDa and corresponds to 20 oocytes. (B) The histogram represents the intensity of the bands analyzed by densitometry. Results are expressed as the relationship between the intensity of protein $p34^{cdc2}$ expression in 20 oocytes and the $p34^{cdc2}$ protein expression in granulose cells (GC). [a,b]Indicate a significant difference in $p34^{cdc2}$ protein expression among diameter groups ($P < 0.05$).

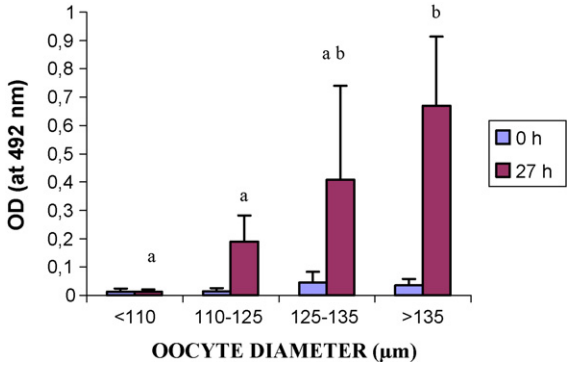


Fig. 4. MPF activity in different sizes of prepubertal goat oocytes at collection time and after IVM. MPF activity is expressed as OD at 492 nm. [a,b]Indicate a significant difference in kinase activity among diameter groups ($P < 0.05$).

MPF activity tended to increase in the biggest oocytes in comparison to the smallest ones ($P < 0.10$). In addition, after IVM, there is a clear and statistically significant increase of MPF activity in the biggest oocytes.

## 4. Discussion

The results of this study show that the oocyte diameter of prepubertal goat oocytes was positively related to the percentage of oocytes reaching metaphase II stage and to the percentage of oocytes developing up to blastocyst stage. Oocyte diameter was also positively related to the amount of $p34^{cdc2}$ protein and MPF activity in prepubertal females. Several studies have examined the relationship between follicle diameter, oocyte diameter and its meiotic and developmental

competence. In goat oocytes, meiotic competence is acquired when oocyte diameter is greater than 136 μm, both in adult [24] and prepubertal females [21]. In the present study, we confirm this fact (Table 2). However, a high proportion of the largest oocytes had resumed meiosis within the follicle (Table 1) and these oocytes, even if they were atresic, presented higher blastocyst development. In bovine oocytes, developmental competence is only adversely affected by advanced atresia [34]. Several studies concluded that oocyte diameter is directly proportional to follicle diameter and as both increase the developmental capability of the oocytes improves in cows (revised by Gandolfi et al. [35]). In adult goats, Crozet et al. [19] found a significant difference in the percentage of blastocysts obtained from oocytes recovered from follicles of 2–3 mm (6%), from follicles of 3.1–5 mm (12%), from follicles >5 mm (26%) and from ovulated oocytes (41%). In our study, the highest percentage of blastocysts (12%) was obtained from oocytes larger than 135 μm recovered from ovaries of prepubertal females. However, larger oocytes from prepubertal goats are recovered mostly from follicles of 2–3 mm [21]. In conclusion, low embryo development of oocytes from prepubertal females is more related to oocyte diameter than to the physiological condition of prepubertal females. In our study, in prepubertal goat oocytes fertilized by IVF, we observed a higher blastocyst rate per cleaved oocyte in oocytes larger than 135 μm (20.45%) compared to oocytes of 125–135 μm (4.92%). However, in our laboratory, using ICSI to fertilize these oocytes categories, we found a blastocyst rate of 11.1 and 15.9%, respectively (Jiménez-Macedo, unpublished data). This difference between oocyte categories after IVF and ICSI protocols could be due to the inability of oocytes of 125–135 μm to be fertilized by IVF, although they were able to develop after a sperm injection. A small percentage of oocytes of 110–125 μm diameter were able to develop up to morulae (6.2%) after ICSI fertilization, but they were unable to develop beyond the 8-cell stage using the IVF protocol. Hyttel et al. [36] reported that in cattle oocytes of 100 μm had full competence for the resumption of meiosis and oocytes of 110 μm had full competence to complete maturation and to sustain embryo development, but a lower number of blastocysts was obtained in these oocytes (30%) in comparison to oocytes larger than 110 μm (60%). Otoi et al. [37], classifying oocytes in six categories according to oocyte diameter concluded that bovine oocytes larger than 115 μm had reached meiotic competence, but to acquire embryo development competence they should have a diameter larger than 120 μm. In conclusion, oocyte size and its relationship to embryo development are different between species.

The synthesis of $p34^{cdc2}$ and Cyclin B1 have been suggested as the limiting factor for oocyte ability to complete nuclear maturation, as was observed studying $p34^{cdc2}$ protein expression in mice [38–41] and goat [32], and Cyclin B1 in pig [42] and cow [43,44]. However, embryo development competence was not analyzed, although Vigneron et al. [45] suggested that expression of $p34^{cdc2}$ and Cyclin B1 could be good markers of embryo development. For this reason, in the present study we have investigated if differences in developmental competence detected in oocytes of different diameter could be related to a differential $p34^{cdc2}$ expression, at mRNA, RNA and protein level, or MPF activity.

To test whether the oocyte developmental competence could be determined by the storage of $p34^{cdc2}$ mRNA in the oocyte, RT-PCR was performed to semi-quantify $p34^{cdc2}$ transcripts with poly-A tail for different oocyte diameters. We observed no differences among the four size groups studied either before or after IVM and $p34^{cdc2}$ mRNA was detected in both competent and incompetent oocytes, as previously described by Dedieu et al. [32] in adult goats and Mitra and Schultz [41] in mice.

The amount of protein in a cell can be regulated by mRNA polyadenylation, as was observed in Xenopus and mice oocytes, where the translation of MPF and MAPK components is regulated by cytoplasmic polyadenylation [46,47]. Poly-A tails are short when the maternal mRNA is stored and they are extended to recruit mRNA for translation [48,49]. Moreover, length of poly-A tails is related to oocyte developmental competence, being shorter in the less competent oocytes [50]. To check possible differences in the stored $p34^{cdc2}$ mRNA we performed the semi-quantification of $p34^{cdc2}$ RNA, because the use of an oligodT primer for the retrotranscription could affect the amount of PCR product detected depending on the polyadenylation state of mRNA [51]. We observed that levels of $p34^{cdc2}$ RNA were higher in oocytes with diameters between 125 and 135 μm than in the other diameters. Possibly, the $p34^{cdc2}$ RNA accumulation in the oocyte takes place during the last growth stage and the translation begins when the oocyte reaches its maximum size, which would explain the decrease of $p34^{cdc2}$ RNA detected in the largest oocytes. Robert et al. [44], who detected a decrease in the levels of Cyclin B1 mRNA (the limiting subunit of MPF in cows) with follicular diameter, also suggested that decrease in mRNA levels was related to the translation of the mRNA into protein to be ready to form the MPF. Despite the differences detected among

diameter groups, the semi-quantification of $p34^{cdc2}$ mRNA and RNA showed no relationship with oocyte developmental competence.

$p34^{cdc2}$ mRNA and RNA levels do not have to be necessarily related to the $p34^{cdc2}$ protein content in the oocyte. For this reason, the study of the expression of the $p34^{cdc2}$ protein as a possible marker of oocyte developmental competence was performed. At collection time, although the corresponding RNA and mRNA were already present in the smallest oocytes, $p34^{cdc2}$ protein was absent from these oocytes. These results were in accordance to previous studies performed in mouse [52] and adult goats [32], which did not detect $p34^{cdc2}$ in incompetent oocytes. However, in other species, $p34^{cdc2}$ was detected in incompetent oocytes (equine: [53]; pig: [54]; rat: [55]), showing that regulation of $p34^{cdc2}$ expression differs between species. Accumulation of $p34^{cdc2}$ increases during oocyte growth (mouse: [52,39]) and during acquisition of meiotic competence (adult goat: [32]; mouse: [39,56]). In contrast, our results did not shown any statistical differences between diameter groups at collection time although $p34^{cdc2}$ was detected in all groups but the smallest ones, probably due to the high variability of our results. We detected the appearance of the protein in the smallest oocytes after 27 h of culture, which clearly shows that the transcript has been translated during the culture period. The appearance of $p34^{cdc2}$ has previously been observed in incompetent oocytes from adult goats after in vitro culture [32]. In our study, the amount of $p34^{cdc2}$ protein detected after in vitro maturation increased with oocyte diameter and was associated with the ability to complete meiosis and to develop into embryos. Dedieu et al. [32] and Chesnel and Eppig [52] also observed an increase in $p34^{cdc2}$ according to the follicle diameter from which oocytes were recovered, in adult goats and mice, respectively. Nevertheless, it is important to note that $p34^{cdc2}$ accumulation during culture was observed for all oocyte diameters, although the $p34^{cdc2}$ levels achieved in the smallest oocytes were lower than those detected in the largest ones. Our results are in accordance to Dedieu et al. [32], who also detected $p34^{cdc2}$ in both competent and incompetent oocytes after in vitro culture, although the accumulation of $p34^{cdc2}$ in the partially competent oocytes was higher, reaching similar levels to fully competent oocytes. However, accumulation of $p34^{cdc2}$ during in vitro culture does not promote the acquisition of meiotic competence in oocytes [52] probably because changes in the ability of $p34^{cdc2}$ and Cyclin B1 to dimerize [56] which depends on Cyclin B1 phosphorylation [57], MPF coincidence in the time with its regulators, such as cdc25 phosphatase or Wee kinases ([53]; reviewed by Jones [58]), or the accumulation of MPF regulators. In fact, in adult goats, the presence of active cdc25C, responsible for dephosphorylation of $p34^{cdc2}$ to activate MPF, is lower in meiotically incompetent than competent oocytes [59]. Therefore, not only the synthesis of $p34^{cdc2}$ protein, but its regulation to become active could also affect the acquisition of oocyte competence. For this reason, it was decided to study MPF activity in addition to $p34^{cdc2}$ expression. In our study, MPF activity was detected in oocytes just after follicle release, when oocytes are supposed to be at the GV stage and, as a consequence, MPF activity should be non-existent in goats [12]. However, we found a high percentage of oocytes at GVBD stage before culture, especially in the largest oocytes, which would explain the detection of MPF activity at collection time. After in vitro maturation, MPF activity increased with oocyte diameter, and it was closely related to oocyte developmental competence in prepubertal oocytes. Our results are in accordance to previous studies that related MPF activity in oocytes with their developmental competence. In fact, Salamone et al. [14] and Ledda et al. [13] reported that MPF activity in prepubertal females was substantially lower than in adult females (cow and ewe, respectively), while Bogliolo et al. [60] reported higher MPF activity in in vivo than in vitro matured oocytes. In addition, higher developmental competence in oocytes treated with caffeine during nuclear transfer has also been related to an increase in MPF activity promoted by caffeine [61].

Bringing together our results of maturation and MPF activity, we observed that, after in vitro maturation, the largest oocytes, which presented the highest MPF activity, also had the highest percentage of oocytes at the metaphase II stage, when the maximum level of MPF activity is detected (mouse: [6–8]; pig: [9]; rabbit: [10]; cattle: [11]; goat: [12]; sheep: [13]). Therefore, it is difficult to establish whether differences in MPF activity among our diameter groups are only caused by differential developmental competence and not by nuclear stage. Nevertheless, as we did not detect differences in the rate of normal fertilization (two pronuclei zygotes) in oocytes bigger than 110 μm, we hypothesis that differences in MPF activity are not only a consequence of different rates in MII oocytes, but to different degrees of cytoplasmic maturation in those oocytes, which is reflected in the differences in blastocyst yield between oocyte diameters. However we have to consider that other factors could affect MPF activity, such as the oocyte's ability to translocate MPF from the cytoplasm to the nucleus (mouse: [62]; cattle: [56]). In summary, we observed a positive relationship between MPF activity ($p34^{cdc2}$ kinase activity), oocyte

diameter and developmental competence, which indicates that MPF activity not only takes part in nuclear maturation, but could also play an important role in cytoplasmic maturation in a direct or indirect way. Nevertheless, more studies on the specific role of MPF in cytoplasmic maturation and its regulation are needed.

In conclusion, our results indicate that the size of oocytes from prepubertal goats is related to their capability to undergo meiotic maturation, in vitro fertilization and to reach the blastocyst stage. Oocyte diameter and, therefore, oocyte developmental competence seems to be related to the amount of $p34^{cdc2}$ protein and MPF activity found in these oocytes after in vitro maturation. MPF activity seems to confer higher developmental competence to oocytes through promotion of cytoplasmic maturation.

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# Total RNA and protein content, Cyclin B1 expression and developmental competence in prepubertal goat oocytes

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**Abstract**

The aim of this study was to examine the relationship between the developmental competence of oocytes and their total RNA and protein contents, and the level of Cyclin B1 transcription. Ovaries from prepubertal goats were collected from a slaughterhouse. Oocytes were recovered by slicing and those with two or more layers of cumulus cells and homogenous cytoplasm were matured in vitro (20 to 25 oocytes per drop) for 27 h. Both before and after IVM, samples of oocytes were denuded and categorised into four group treatments by diameter (< 110 µm, 110 to 125 µm, 125 to 135 µm; > 135 µm), separated into sub-groups of 10 oocytes per treatment-replicate and stored in liquid nitrogen until total RNA content analysis by spectophotometry, total protein content analysis by a colorimetric assay and Cyclin B1 transcription analysis by RT-PCR. For the study of developmental competence, the rest of the matured oocytes were fertilised in vitro in groups of 20 to 25 for 24 h. Presumptive zygotes were denuded, sorted into the four categories of diameter noted above, and placed into culture drops in groups of 18 to 25 for in vitro culture. Cleavage rate was evaluated at 48 hpi and embryo development at 8 d post-insemination. There were four replicates of each treatment for each assay or evaluation point of the experiment. There were no significant differences between the size categories of oocytes at collection in total RNA content, total protein content and Cyclin B1 mRNA. There were significant differences ($P < 0.05$) in the expression of Cyclin B1 before IVM with oocytes in the > 135 mm diameter category having the highest value for this variant. There were no significant differences in these characteristics between the categories of oocyte diameter after IVM except in respect of total RNA content, which was lower for the largest size of oocytes (>135 µm; mean ± SD = 12.3 ± 1.84 ng/oocyte) than the other three size groups (19.2 ± 1.38 to 22.1 ± 4.44 ng/oocyte; $P < 0.05$). Significant differences ($P < 0.05$) in cleavage rate were observed between the different oocyte size categories (<110 µm, 3.0 %; 110 to 125 µm, 32 %; 125 to 135 µm, 50 %; >135 µm, 73 %). Only oocytes >125 µm diameter developed to the blastocyst stage (125 to 135 µm, 7 %; >135 µm, 10 %). This study showed that the RNA content and the Cyclin B1 RNA expression of prepubertal goat oocytes, and their development to embryos varied between the different size categories of the oocytes.

*Keywords:* Cyclin B1, MPF, goat, oocyte, diameter

## 1. Introduction

Some studies have demonstrated that the low blastocyst rate obtained *in vitro* is mainly due to insufficient cytoplasmic maturation in the oocytes, which is reflected in the inability of the fertilized oocyte to decondense the sperm head, to block polyspermy, or inability to maintain embryo development beyond the transition of embryonic genome activation (Sirard et al., 2006). In addition, problems related to insufficient cytoplasmic maturation seem to be higher in oocytes obtained from prepubertal than adult females (Armstrong, 2001). Results from previous studies in our laboratory also indicate a deficient cytoplasmic maturation in oocytes recovered from prepubertal goats at the slaughterhouse, a low penetration rate (Urdaneta et al., 2004), different distributions of cortical granules (Velilla et al., 2004) and mitochondria (Velilla et al., 2006) when compared to adult goats, and a low blastocyst rate (Jimenez-Macedo et al., 2005; Urdaneta et al., 2004). The small follicular diameter of prepubertal goat ovaries, most of them between 2.5 to 3 mm (Martino et al., 1994), makes it necessary to recover the oocytes by slicing the ovarian surface, which thus gives a high number of oocytes. However, the oocyte population recovered is highly heterogeneous and the oocytes have different maturational status and different degrees of atresia. Oocyte selection made so far in our laboratory, based on cumulus-oocyte complex (COC) morphology, seemed not to be good enough to distinguish between developmentally competent and incompetent oocytes due to the low blastocyst numbers obtained. In adult goats, Crozet et al. (1995) found a direct and positive relationship between follicular diameter and embryo development, concluding that follicles bigger than 5 mm contain oocytes which have a better competence to develop up to blastocyst stage. In prepubertal goat ovaries, follicles larger than 5 mm in diameter are practically non-existent (Martino et al., 1995). In both adult (De Smedt et al., 1994) and prepubertal (Martino et al., 1994) goat oocytes meiotic oocyte competence has been classified in terms of the oocyte diameter as: <110 µm, corresponding to incompetent oocytes, 110 to 125 µm, corresponding to partially competent oocytes, 125 to 135 µm and > 135 µm, corresponding to competent oocytes. A study performed in our laboratory comparing embryo development between different diameters of prepubertal goat oocytes showed a

higher blastocyst rate in oocytes with a diameter higher than 135 µm (12.5 %; Anguita et al., 2006).

During the oocyte growth phase, synthesis and storage of proteins and heterogeneous RNA take place (Crozet et al., 1981). This storage is necessary to maintain early embryonic development until the activation of the embryonic genome (Bachvarova, 1992). It is possible that the low rate of *in vitro* development could be explained by an insufficient storage of proteins and RNA in the oocyte cytoplasm, maybe because they are recovered for IVM when they have not yet finished the growth phase.

Oocyte meiotic competence is described as its ability to resume meiosis and progress to metaphase II (MII) stage, where the oocyte remains in arrest until fertilization. Meiotic maturation is regulated by several protein kinases, and MPF is considered the most important. MPF is a heterodimer composed of a $p34^{cdc2}$ catalytic subunit, with serine-threonine kinase activity, and a Cyclin B1 regulatory subunit (Labbe et al., 1989). MPF activity has been described in many mammalian oocytes: it appears just before GVBD and increase until metaphase I stage, then its activity decreases in anaphase-telophase and increases again, reaching its maximum level in metaphase II (mouse: Fulka et al., 1992; Hashimoto and Kishimoto, 1986; Verlhac et al., 1994; pig: Naito and Toyoda, 1991; rabbit: Jelinkova et al., 1994; cattle: Collas et al., 1993; goat: Dedieu et al., 1996; sheep: Ledda et al., 2001). The limiting factor for MPF activation has been studied in several species and species-specific differences have been identified (mouse: Chesnel and Eppig, 1995; Mitra and Schultz, 1996; pigs: Naito et al., 1995; Sun et al., 2001; goat: Dedieu et al., 1998; cattle: Levesque and Sirard, 1996; Robert et al., 2002). Moreover, MPF activity has been shown to be lower in prepubertal than in adult female oocytes (cow: Salamone et al., 2001; sheep: Ledda et al., 2001). Therefore, low developmental competence could be explained by a deficiency of one of the MPF subunits.

The objective of this study was to analyze the possible relationship between total RNA and protein content, Cyclin B1 mRNA and RNA expression in prepubertal

goat oocytes with oocyte developmental competence. To this purpose, analyses were performed in oocytes of different diameter.

## 2. Material and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### *2.1. Oocyte collection and IVM*

Ovaries from prepubertal goats, approximately 2 months old, were recovered from a local slaughterhouse and transported within 2 hours to the laboratory at 38.5 ºC in PBS (Solution Dulbecco's phosphate-buffered saline, P-4417) which contained 50 µg/mL gentamycin (G-3632). Ovaries were washed three times in PBS containing gentamycin, and COCs (cumulus-oocyte complexes) were recovered by slicing in a 60 mm culture dish containing TCM199 (M-2520) supplemented with 2.2 mg/mL $NaHCO_3$, 2% (v/v) steer serum (Donor Bovine Serum®, CanSera, Canada) and 50 µg/mL gentamycin. Only oocytes with two or more layers of cumulus cells and homogeneous cytoplasm were used for IVM.

COCs were matured in TCM199 medium (M-7528) supplemented with 275 µg/mL sodium pyruvate (P-3662), 146 µg/mL L-glutamine (G-5763), 10% (v/v) steer serum, 10 µg/mL o-LH (L-5269), 10 µg/mL o-FSH (F-4520), 1 µg/mL 17-β estradiol (E-2257), 400 µM cysteamine (M-9768) and 50 µg/mL gentamycin (G-3632). Groups of 20-25 COCs were transferred to 100 µl microdrops of maturation medium under mineral oil (M-3516) and incubated for 27 hours at 38.5 ºC in a humidified air atmosphere with 5% $CO_2$.

### *2.2. Sperm capacitation and IVF*

Matured oocytes were fertilized with fresh semen collected from two goats of proven fertility. Ejaculates were collected with artificial vaginas and

transported to the laboratory at 38.5 ºC within 30 minutes. Ejaculates were mixed and the motile sperm fraction was separated by the swim-up method (Parrish et al., 1986). Briefly, 70 µL of sperm was placed at the bottom of three conic tubes containing 2 mL of mDM (Defined medium (Brackett and Oliphant, 1975), modified by Younis et al. (1991)), and it was incubated for 45 min at 38.5 ºC in a humidified air atmosphere with 5% $CO_2$. After incubation, 600 µL of the supernatant of each tube was recovered and centrifuged at 170 X g for 3 minutes. After discarding the supernatant, the pellet was resuspended in mDM (1:1; v/v) containing heparin (H-3149) and ionomycin (I-0634) (Final concentration: 10 µg/mL heparin and 200 nM of ionomycin) and incubated for 15 min in a humidified air atmosphere with 5%$CO_2$ and 38.5 ºC (Wang et al., 2002). Sperm concentration was determined using a Neubauer chamber cell.

After maturation, groups of 20 to 25 oocytes were transferred to 100 µL microdrops of modified Tyrode's medium (TALP) as described by Parrish et al. (Parrish et al., 1986), supplemented with 1 µg/mL hypotaurine (H-1384) and 0.3 mg/mL glutathione (G-6013) under mineral oil. Oocytes were co-cultured with capacitated spermatozoa in a final concentration in the microdrop of 4 x $10^6$ sperm cells/mL.

*2.3. In vitro embryo culture*

At 24 hours after insemination (hpi), presumptive zygotes were washed in SOF medium (Synthetic Oviductal Fluid, (Holm et al., 1999)) and denuded by pipetting to separate them from sperm cells. Groups of 18-25 embryos were placed to 25 µL microdrops of SOF medium in 35 mm culture dishes under mineral oil, and they were cultured in a humidified atmosphere with 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5 ºC for 7 days. At 48 hpi cleavage rate was evaluated and 0.1 µL fetal bovine serum (F-7524) per embryo was added to the medium. At the end of the culture period (8 days post-insemination), total cell number of embryos was evaluated by Hoechst 33342 staining (B-2261) under a fluorescence microscope.

*2.4. RNA extraction*

Total RNA from 10 oocytes was extracted using 100 µL of TriReagent® (T-9424). We added 20 µg of glycogen (92760323, Roche, Germany) to the 100 µL of TriReagent® as a carrier for the RNA precipitation and 1 pg of rabbit globin mRNA (R-1253) per oocyte that was used as an extrinsic control of the whole process. After adding 20 µL of chloroform, we centrifuged the sample at 12000 X g for 20 min at 4 ºC. The aqueous phase was recovered and the RNA was precipitated with 60 µL of isopropanol at -20 ºC overnight, while the resulting organic phase was frozen at -80 ºC until its use in protein extraction. The next day, after centrifugation at 12000 X g at 4 ºC for 20 min, the supernatant was discarded and the pellet was washed sequentially with 70% ethanol and 100% ethanol. For the washing steps, the sample was centrifuged at 12000 X g at 4 ºC for 15 min. The pellet was dried and resuspended in 4 µL of $H_2O$ DEPC.

The RNA extracted was incubated for 30 min at 37 ºC with 1U/µL DNase (18047-019, Invitrogen Corporation, USA), and stored at -80 ºC if the RT-PCR or the RNA quantification could not be done immediately.

*2.5. Total RNA quantification*

Total RNA obtained after RNA extraction and DNA digestion was quantified by spectrophotometry, in a wavelength of 260 nm. A known amount of goat ovary RNA was used as an efficiency control of the process.

*2.6. Protein extraction and protein quantification*

Protein from 40 oocytes was extracted using TriReagent® (T-9424). DNA was precipitated with 30 µL of 100% ethanol and centrifuged at 12000 X g at 4 ºC for 5 min. The supernatant was recovered and then transferred to a new tube. Proteins were precipitated with 150 µL of isopropanol for 10 min at room temperature, and centrifuged at 12000 X g at 4 ºC for 10 min. The supernatant was discarded, and the pellet was washed three times with 200 µL of 0.3 M guanidine hydrochloride/95% ethanol solution. During each wash, samples were stored in

wash solution for 20 min at room temperature. Samples were centrifuged at 7500 X g for 5 min at 4ºC. After the three washes, we added 1 mL of 100% ethanol and vortered the protein pellet. The sample was left at room temperature for 20 min, and centrifuged at 7500 X g for 5 min at 4 ºC. We discarded the supernatant, and the protein pellet was dried for 5-10 min. The pellet was dissolved in 20 µL of 1% SDS, and stored at -80 ºC until its use for quantification.

Total protein quantification of 5 µL of the protein extract (corresponding to 10 oocytes) was performed with *DC Protein Assay* (BioRad, USA), following the manufacturer's instructions. This is a colorimetric assay, and the color developed in each reaction is proportional to the protein content of the sample. Different concentrations of BSA (0, 50, 100, 200, 300, 400, 500, 600, 700, 1000 and 1410 µg/mL) diluted in SDS 1% were used as the control. The developed color of each reaction was measured in a microplate ELISA reader (iEMS Reader, LabSystems) at 690 nm.

*2.7. Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)*

In order to know Cyclin B1 mRNA and RNA expression in prepubertal goat oocytes according to their diameter, for each size group total RNA corresponding to 10 oocytes was reverse-transcribed with *ThermoScript™ RT-PCR System* (Invitrogen Corporation, USA), following the manufacturer's instruction, in a final volume of 20 µL. The RT was performed using oligodT and random primers. Four µL of these reactions were used to amplify Cyclin B1.

To perform Cyclin B1 amplification, cDNA was incubated with 1 X PCR buffer, 1 µM of each primer, 2 mM of $MgCl_2$, 200 µM of each dNTP and 0.1 U/ µL of Taq polymerase GoldStar (ME-0064-01, Eurogentec, Belgium) in a final volume of reaction of 25 µL for 34 cycles (94 ºC 1 min, 58 ºC 1 min, 72 ºC 1 min) in a thermocycler 9700 (Applied BioSystems, USA). The Cyclin B1 sense and antisense primers were the following: 5'-GAGCCATCCTCATTGACTGGC and 5'-CTTAGATGCTCTCCGAAGG (Hue et al., 1997).

To perform globin amplification, cDNA was incubated with 1 X PCR buffer, 0.5 µM of each primer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP and 0.1 U/µL of Taq polymerase GoldStar in a final volume of 25 µL for 30 cycles. Globin PCR conditions were determined by the amplification conditions of Cyclin B1. The globin sense and antisense primers were the following: 5'-GCAGCCACGGTGGCGAGTAT and 5'-GTGGGACAGGAGCTTGAAAT (Knijn et al., 2002) .

At the same time, a PCR without cDNA and a PCR of the RT product performed without RNA were used as negative controls. In no case was an amplification band detected.

Amplification bands were visualized in a 2% agarose gel stained with ethidium bromide. The weight marker band (P-9577) corresponding to 300 pb was used as a control to avoid light variations in the exposition to UV. Each amplification band was quantified by densitometric analysis using the PC program *Quantity One* (Bio-Rad Laboratories, USA). The results were normalized with the quantification of globin amplification band. Direct sequencing of the amplifications products was performed using an ABI-prism DNA sequencer.

*2.8. Experimental design*

All the oocytes used for each replicate and assay were collected at the same day. Four replicates of each assay were performed. Each replicate was started at a separate day.

Before IVM, a sample of oocytes selected at random was denuded in PBS plus 300 µg hyaluronidase/mL (H-4272), measured at X 96 excluding the zona pellucida and classified by diameters in four categories: <110 µm, 110 to 125 µm, 125 to 135 µm and >135 µm according to the criteria described by Anguita et al. (2006) in prepubertal goats. From each diameter category, at least four sub-samples each of 10 oocytes were washed, frozen in liquid nitrogen and stored at -80 ºC until its use in one of the four analyses. The remainder of the oocytes, still not separated by diameter categories, were matured in vitro. After 27 h of in vitro maturation, a

sample of oocytes were denuded, measured and classified by diameters as described above. At least four sub-samples of 10 oocytes of each category were washed and stored as described above until its use in one of the four analyses. The remainder of oocytes was fertilized in vitro. At 24 hpi, the presumptive zygotes were measured and classified in the four categories and culture in vitro for additional 7 days. Each drop contained 18-25 embryos of the same diameter category.

RNA extraction was performed in four sub-samples of 10 oocytes of each replicate and size category. The RNA corresponding to one sub-sample per size category and replicate was used to perform total RNA content and Cyclin B1 transcription analyses. The organic phase obtained in the RNA extraction of the four sub-samples of each size category (40 oocytes per category) and replicate was stored at -80ºC until its use in protein extraction and quantification.

2.9. Statistical analysis

Embryo development, PCR semi-quantification, total RNA quantification and total protein quantification results of different diameter groups were compared with a One-Way ANOVA test with Tukey's post-test by the PROC MIXED procedure of SAS (Version 9.2, Inst., Inc, Cary, NC). Data from embryo development were transformed using arcsine square root before statistical analysis. Each oocyte size group was represented by four replicates for each of the variates studied. A value of $P < 0.05$ was accepted as the minimum level of significance in all the statistical tests. Data are presented as percentage ± SD.

## 3. Results

### *3.1. Embryonic development*

Results of embryonic development of different size oocytes from prepubertal goats are shown in table 1. Total number of embryos obtained increased with oocyte diameter, and there were significant differences among the four diameter

groups (<110 µm: 3%; 110 to 125 µm: 32%; 125 to 135 µm: 50%; >135 µm: 73%). Blastocyst rate in 125-135 µm oocytes (7%) and > 135 µm oocytes (10%) was significantly higher than in <125 µm oocytes (0%) ($P < 0.05$).

### *3.2. Total RNA quantification*

For each diameter group, total RNA corresponding to 10 oocytes, before and after IVM, was quantified by spectrophometry at 260 nm. Results, expressed as ng/oocyte, are shown in table 2. As we can observe, we detected no statistically significant differences among the four categories of diameters before IVM (<110 µm: 22.9 ± 6.5; 110 to 125 µm: 21.9 ± 6.8; 125 to 135 µm: 19.7 ± 6.5; >135 µm: 26.5 ± 11.1) ($P>0.05$). However, after IVM, the total RNA detected was lower in the biggest oocytes (>135 µm: 12.3 ± 1.8) when compared to oocytes 110-125 µm oocytes (22.1 ± 4.4), and oocytes from 125 to 135 µm (19.2 ± 1.4) ($P=0.08$).

### *3.3. Total protein quantification*

Total protein oocyte quantification from each size group was performed using the DC Protein Assay. As we can observe in table 3, we detected no statistically significant differences between the four groups of size at any of the hours analyzed.

### *3.4. Cyclin B1 mRNA and RNA expression*

Results of Cyclin B1 mRNA expression at collection time and after IVM are represented in figure 1. No statistically significant differences in Cyclin B1 mRNA expression were detected among the four groups of oocyte size before and after IVM. However, the expression of Cyclin B1 mRNA at collection increased as oocyte diameter increased from 0.50 ± 0.07 for oocytes < 110 µm to 0.96 ± 0.33 for 110 to 125 µm oocytes, but declined with further increase in diameter (125 to 135 µm: 0.86 ± 0.33; >135 µm: 0.58 ± 0.37).

Results of Cyclin B1 RNA expression are shown in figure 2. Before IVM, Cyclin B1 RNA expression was highest in the largest oocytes (<110 µm: 0.23 ± 0.13; 110 to 125 µm: 0.59 ± 0.11; 125 to 135 µm: 0.53 ± 0.54; >135 µm: 1.27 ± 0.78) (P=0.04). However, after IVM no statistically significant differences were detected among size categories. The expression of Cyclin B1 RNA increased from collection to the completion of IVM for oocytes < 125 µm (P < 0.05), 110 to 125 µm (P < 0.05) and 125 to 135 µm (P < 0.10), but decreased for oocytes > 135 µm diameter (NS).

## 4. Discussion

The characteristics that determine whether an oocyte is competent to develop into an embryo are still unknown. It seems that cytoplasmic oocyte maturation is the key to a better understanding of oocyte developmental competence. However, processes that take place during that maturation and, most importantly, their regulation, are still poorly understood. The aim of our study was to try to clarify how the storage and expression of certain molecules that could be related to cytoplasmic oocyte maturation differ between oocytes with different developmental competence.

We have observed that oocyte developmental competence in prepubertal goat oocytes is related to the diameter; oocytes smaller than 125 µm were not able to develop to blastocyst stage and a maximum blastocyst rate was reached in oocytes with a diameter larger than 135 µm (10%), but there were no differences when compared to oocytes from 125 to 135 µm. The capacity of prepubertal goat oocytes to reach blastocyst stage was previously study in our laboratory, and we observed for the first time that prepubertal goat oocytes with a diameter higher than 125 µm were able to develop to blastocyst stage (Anguita et al., 2006). Moreover, we observed that the inability of oocytes smaller than 125 µm to maintain embryonic development was due to a deficient nuclear maturation in these oocytes, and only 0 and 20.7% of oocytes reached metaphase II stage after IVM in oocytes smaller than 110 µm and oocytes form 110- to 125 µm, respectively (Anguita et al., 2006). In this earlier study, we also observed that oocytes from 110 to 125 µm which completed nuclear maturation were able to be normally fertilized

(2PN = 28.8%), showing no differences compared to oocytes higher than 125 µm, but they were unable to surpass the 2-8 cell stage in embryonic development, which may indicate that these oocytes have not stored enough RNA and proteins to reach blastocyst stage. The relationship between follicular size, oocyte diameter and developmental competence has been observed in many species (goat: Crozet et al., 1995; cow: Kauffold et al., 2005; Lequarre et al., 2005; Otoi et al., 1997; pig: Marchal et al., 2002; buffalo: Raghu et al., 2002). In adult goats, Crozet et al. (1995) observed a significant difference in the percentage of blastocysts obtained from oocytes recovered from follicles of 2 to 3 mm (6%), from follicles of 3.1 to 5 mm (12%), from follicles bigger than 5 mm (26%) and from ovulated oocytes (41%). Crozet et al. (2000) described the relationship between goat oocyte diameter and follicle diameter in adult goats. Thus, oocytes with a mean diameter of 96 µm were recovered from follicles smaller than 0.5 mm; oocytes of 120 µm were recovered from follicles of 0.5 to 0.8 mm; oocytes of 125 µm from follicles of 1 to 1.8 mm; oocytes of 136 µm (range 125 to 146 µm) from follicles of 2 to 3 mm and oocytes ranging from 130 to 146 µm from follicles larger than 3 mm. In the present study, we obtained a higher blastocyst rate in 125 to 135 µm oocytes (7%) than in a previous study (1.95%; Anguita et al., 2006). This improvement could be due to the use of glutathione (GSH) during fertilization in vitro. Urdaneta et al. (2004) observed an increase in normal fertilization (zygotes with two pronuclei) and cleavage when GSH was used in in vitro fertilization of prepubertal goat oocytes. Also, an increase in blastocyst rate has been reported when glutathione was used in the IVF medium (pigs: Boquest et al., 1999; cattle: Van Soom et al., 1998). Thus, GSH plays an important role as cellular protection during oxidative stress and helping cell proliferation during embryonic events (Del Corso et al., 1994; Lafleur et al., 1994; Yu, 1994). These results seem to indicate that the improvement in blastocyst rate detected in oocytes with 125-135 µm diameters in comparison to the results of Anguita et al. (2006) could be related to a beneficial effect of the GSH during fertilization.

During oocyte growth in vivo, RNA and protein storage take place (Gosden et al., 1997). RNA synthesis ceases when oocytes resume meiosis (Hyttel et al., 2001). Meiotic progression up to MII, fertilization and early embryonic development must

be maintained with the stored RNA and proteins (Bachvarova, 1992). Deficient storage of RNA and proteins, therefore, could lead to a decrease in the developmental competence of the oocyte. To check if the differences in developmental competence between different diameters of prepubertal goat oocytes could be explained by a differential storage of RNA or proteins, we performed total RNA and protein quantification before and after IVM. We observed that the total RNA stored before IVM in the oocytes was not related to oocyte size. After maturation, total RNA decreased in the largest oocytes, perhaps due to a higher capability of these oocytes to mobilize the stored RNA and to synthesize new proteins, which could explain their high developmental competence. However, no significant differences in total protein content were observed between the different oocyte diameters before or after maturation. Therefore, we could not determine whether the lower RNA content detected in the biggest oocytes is due to a mobilization of stored RNA or not.

MPF is the main meiotic regulator, and it is thought to be a possible cytoplasmic maturation regulator too. It is composed of two subunits, the catalytic subunit $p34^{cdc2}$ and the regulatory subunit Cyclin B1 (Labbe et al., 1989). It has been suggested that the oocytes inability to resume or complete meiosis could be related to deficient MPF activation or the lack of one of the MPF subunits, but the limiting factor varies among species: in the mouse, meiotic competence acquisition is related to $p34^{cdc2}$ protein synthesis (Chesnel and Eppig, 1995; De Vantéry et al., 1996; de Vantery et al., 1997; Mitra and Schultz, 1996); in adult goats $p34^{cdc2}$ is considered the key factor for meiotic resumption (Crozet et al., 2000; Dedieu et al., 1998); and in pigs (Sun et al., 2001) and cattle (Levesque and Sirard, 1996) the limiting factor for meiotic resumption is the Cyclin B1 subunit. As observed, many studies have tried to establish a relationship between MPF subunit expression and oocyte meiotic competence, but we have not found any study of MPF expression related to developmental competence, although Vigneron et al. (2004) suggested that the expression of $p34^{cdc2}$ and Cyclin B1 could be good markers of embryo development. In our study, we have analyzed Cyclin B1 expression at a transcriptional level by RT-PCR. We observed no differences in Cyclin B1 mRNA expression in the four groups of size before or after IVM. It is important to notice

that Cyclin B1 mRNA was detected in all the diameters evaluated, what is in accordance with the results obtained by Hue et al. (1997) who detected Cyclin B1 mRNA in both meiotically competent and incompetent oocytes. Although it is not statistically different, before IVM Cyclin B1 mRNA expression seemed to increase in oocytes with diameters from 110 to 135 µm, possibly due to the accumulation of the mRNA that takes place during the last growth phase in the oocytes (Gosden et al., 1997). The decrease of Cyclin B1 mRNA observed in the biggest oocytes could indicate the use of that mRNA to synthesize the Cyclin B1 protein needed to associate with $p34^{cdc2}$ to form pre-MPF. In fact, in an earlier study we observed that most oocytes had resumed meiosis at collection time, and the percentage of GVBD-oocytes increased with oocyte diameter (<110 µm: 5.33 %; 110 to 125 µm: 26.66 %; 125 to 135 µm: 64.40 %; >135 µm: 80.30 %; Anguita et al., 2006). The association of Cyclin B1 and $p34^{cdc2}$ and its subsequent activation is a requirement for meiosis resumption in many mammalian oocytes (mouse: Chesnel and Eppig, 1995; Choi et al., 1991; pig: Christmann et al., 1994; cattle: Wu et al., 1997; rabbit: Jelinkova et al., 1994; goat: Dedieu et al., 1996). Our results concur with our previous study which detected a positive relationship between the developmental competence of prepubertal goat oocytes and diameters, higher $p34^{cdc2}$ protein content and MPF activity (Anguita et al., 2006). As MPF requires not only the synthesis of $p34^{cdc2}$, but also of Cyclin B1, the present results suggest that the decrease of Cyclin B1 mRNA in the biggest oocytes and the supposed Cyclin B1 synthesis could lead to an increase in MPF formation and activity, which could also be related to the higher developmental competence of these oocytes.

In addition to Cyclin B1 mRNA expression, we also evaluated Cyclin B1 RNA expression by RT-PCR using random primers instead of oligo-dT primers. The protein amount in a cell can be regulated by mRNA polyadenylation, as was observed in Xenopus and mice oocytes, where translation of MPF and MAPK components is regulated by cytoplasmic polyadenylation (Xenopus: Charlesworth et al., 2000; de Moor and Richter, 1999; mouse: Krischek and Meinecke, 2002). Poli-A tails are short when the maternal mRNA is stored and they are extended to recruit mRNA for translation (Huarte et al., 1992; Temeles and Schultz, 1997). As a

consequence, the use of oligo-dT primers could affect the PCR product detected depending on the polyadenylation state of mRNA (Lequarre et al., 1997).

Cyclin B1 RNA expression increased with increasing oocyte diameter before IVM, but not after maturation. After IVM, there were no significant differences between the treatments, probably due to the Cyclin B1 RNA storage that takes place during maturation in oocytes smaller than 135 µm. In the largest oocytes, however, Cyclin B1 expression during maturation did not increase, probably due to the higher RNA consumption rate of the larger oocytes in comparison to the other size groups. Although the rest of size groups reach the levels of the biggest group, they did not reach the blastocyst rate achieved by the oocytes bigger than 135 µm. These results could suggest that the amount of stored Cyclin B1 RNA at oocyte collection time is related to oocyte size as well as to meiotic and developmental competence. These results also suggest the possible role that Cyclin B1 plays not only in oocyte nuclear maturation but, also in cytoplasmic maturation.

## 5. Conclusion

In conclusion, under the conditions of the present study, development to the blastocyst stage of prepubertal goat oocytes depended upon the denuded oocytes having a minimum diameter of 125 µm. This study showed that the RNA content and the Cyclin B1 RNA expression of prepubertal goat oocytes, and their development to embryos, varied between the different size categories of the oocytes.

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Table 1: In vitro embryo development up to 8 days post-insemination of prepubertal goat oocytes of different diameters.

| | <110 μm | 110 to 125 μm | 125 to 135 μm | >135 μm |
|---|---|---|---|---|
| Oocytes inseminated | 25 | 52 | 108 | 61 |
| Oocytes cleaved* (% ± SD) | 3 ± 6 c | 32 ± 16 b | 50 ± 12 ab | 73 ± 15 a |
| 2-7 cells* (% ± SD) | 3 ± 6 b | 18 ± 6 a | 33 ± 8 a | 47 ± 24 a |
| 8-16 cells* (% ± SD) | 0 b | 9 ± 8 a | 8 ± 3 a | 10 ± 4 a |
| Morulae* (% ± SD) | 0 | 5 ± 6 | 2 ± 3 | 5 ± 3 |
| Blastocysts* (% ± SD) | 0 b | 0 b | 7 ± 3 a | 10 ± 9 a |

a, b, c, d Values within a line with different letters are significantly different ($P < 0.05$)

* Results are expressed as the mean percentage of embryos obtained in each classification in relation to the total number of embryos analyzed. These data were transformed using arcsine square root to perform statistical analysis.

Table 2. Total RNA in prepubertal goat oocytes of different diameters before and after in vitro maturation.

| IVM | <110 μm (ng/oocyte ±SD) | 110 to 125 μm (ng/oocyte ±SD) | 125 to 135 μm (ng/oocyte ±SD) | >135 μm (ng/oocyte ±SD) |
|---|---|---|---|---|
| 0 h | 22.9 ± 6.5 | 21.9 ± 6.8 | 19.7 ± 6.5 | 26.5 ± 11.1 |
| 27 h | 21.1 ± 7.0 ab | 22.1 ± 4.4 a | 19.2 ± 1.4 a | 12.3 ± 1.8 b |

SD: Standard Deviation

a, b Values within a line with different letters are significantly different ($P < 0.05$)

Table 3. Total protein content of prepubertal goat oocytes of different diameters (values are for 10 oocytes) before and after in vitro maturation

| IVM | <110 μm (μg/ml ±SD) | 110 to 125 μm (μg/ml ±SD) | 125 to 135 μm (μg/ml ±SD) | >135 μm (μg/ml ±SD) |
|---|---|---|---|---|
| 0 h | 734 ± 255 | 573 ± 158 | 815 ± 221 | 787 ± 135 |
| 27 h | 772 ± 197 | 761 ± 167 | 804 ± 295 | 611 ± 180 |

SD: Standard deviation

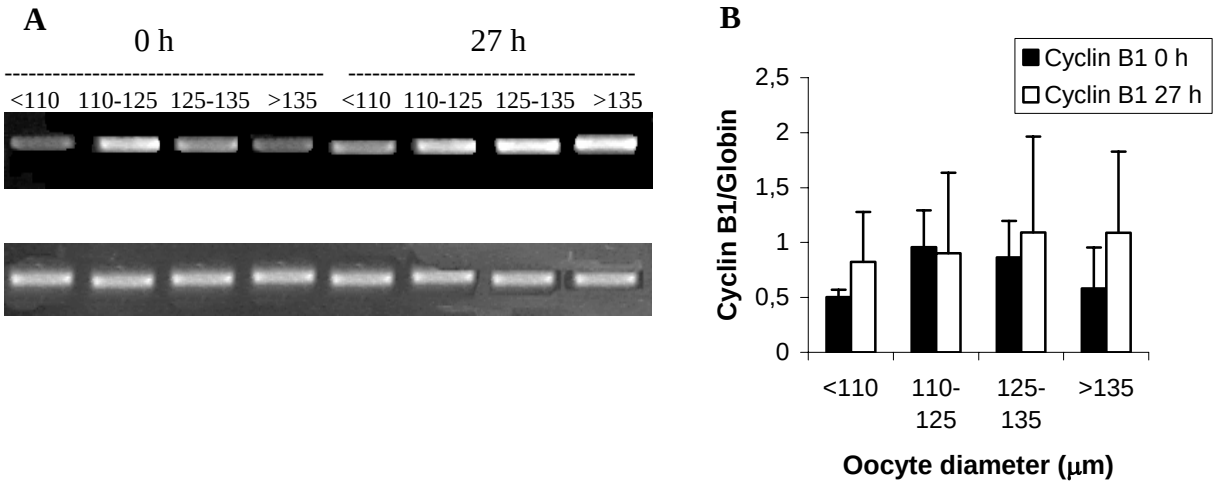


Figure 1. Cyclin B1 mRNA expression in different diameters of prepubertal goat oocytes at collection (0h) and after IVM (27h). **A)** The Cyclin B1 RT-PCR product corresponding to 10 oocytes for each diameter group (upper band) and their corresponding rabbit globin control (lower band). B) The histogram represents the intensity of the bands analyzed by densitometry. Results are expressed as the relationship between the intensity of Cyclin B1 mRNA expression band and mRNA rabbit globin band.

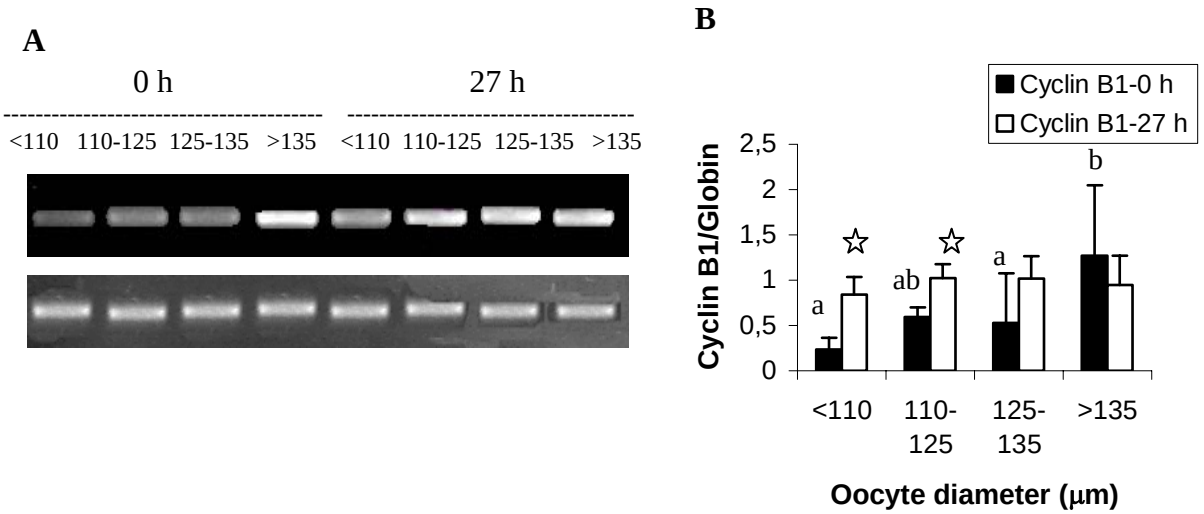


Figure 2. Cyclin B1 RNA expression in different diameters of prepubertal goat oocytes at collection (0h) and after IVM (27h). **A)** The Cyclin B1 RT-PCR product corresponding to 10 oocytes for each diameter group (upper band) and their corresponding rabbit globin control (lower band). **B)** The histogram represents the intensity of the bands analyzed by densitometry. Results are expressed as the relationship between the intensity of Cyclin B1 RNA expression band and mRNA rabbit globin band.

a, b Values in the histogram with different letters indicates significantly differences among size categories within the same time analyzed ($P < 0.05$).

☆ symbol in the histogram indicates significantly differences among the values obtained at different hours (0 or 27 h) within the same size category ($P < 0.05$).

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# Developmental competence of bovine oocytes is not related to apoptosis incidence in oocytes, cumulus cells and blastocysts

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**Abstract**

The number of follicles undergoing atresia in an ovary is very high, and isolation of cumulus–oocyte complexes (COCs) from such atretic follicles may impair subsequent embryo development in vitro. Our aim was to study if stringent selection by morphological assessment of COCs can improve embryo development, and to evaluate whether oocyte diameter is related with apoptotic ratio in oocytes and blastocysts. COCs from slaughtered cattle were recovered by follicle aspiration and classified depending on oocyte diameter: (A) <110 μm; (B) 110–120 μm; (C) >120 μm. COCs were matured, fertilized and cultured in vitro. Early and late stages of apoptosis were detected by Annexin-V and TUNEL staining, respectively, in denuded oocytes, COCs and blastocysts. Immature oocytes from Group A showed higher apoptotic ratio assessed by TUNEL assay, and the COCs corresponding to this group also showed a higher proportion of apoptotic cumulus cells. After maturation, no differences were present in the incidence of apoptosis among oocytes from different groups, but COCs corresponding to the largest diameter showed less apoptotic cumulus cells. In addition, the percentage of apoptotic oocytes decreased during in vitro maturation in all groups. Apoptotic cell ratio (ACR) in blastocysts was not related to oocyte diameter. In conclusion, oocyte selection and oocyte morphological evaluation prior to maturation was not sufficient to select non-atretic oocytes. When oocyte diameter was used as an additional selection the embryonic developmental potential increased together with oocyte diameter, but this improvement was not related to a lower incidence of apoptosis in the largest oocytes.


*Keywords:* Apoptosis; Bovine; Oocyte; Cumulus; Blastocyst

## 1. Introduction

It is generally accepted that in vitro embryo production is very inefficient when compared to in vivo embryo production in terms of blastocyst rate [1,2] and pregnancies obtained [2–5]. The efficiency of bovine embryo production in vitro, measured as the blastocyst rate obtained from oocytes matured and fertilized in vitro, is about 40% (reviewed by ref. [6]). This reduced blastocyst yield obtained in vitro seems to be related to oocyte source, while the quality of the embryos produced in vitro, measured in terms of survival of embryos after cryopreservation [7,8], embryo development speed and specific gene expression [8] is related to the culture system applied (reviewed by ref. [9]).

We understand oocyte quality as the characteristics that confer the oocyte capability to develop into an embryo, establish a pregnancy and produce healthy offspring. The best indicator of oocyte quality would be the rate of born animals generated per transferred embryo; but because it is very difficult to transfer each

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embryo obtained, oocyte quality is generally only evaluated by means of blastocyst rate. Developmental competence in the oocyte is achieved during their growth in the follicle (reviewed by ref. [10]) when accumulation of transcripts and proteins takes place [11]. Storage and mobilization of these molecules is necessary to maintain embryonic development until the maternal to zygotic transition stage [12,13], and to activate the embryonic genome expression through the stored maternal transcription factors [14]. Many approaches have been performed in order to find out which are the characteristics that affect oocyte quality: mitochondrial content of the oocyte [15,16] detected by PCR; differential relative abundance of specific transcripts in the oocyte [17,18] or in the embryo [19,20] detected by RT-PCR; transcriptional activity [21] detected by radio-labelling; apoptosis incidence in embryos [22–24], in oocytes [23–25] or in cumulus cells [25–28] detected by TUNEL staining, expression of pro- and anti-apoptotic molecules (such as Bax, Bcl-2, respectively), or caspase activity analysis. The study of apoptosis and its influence in oocyte and embryo quality has great relevance, especially in in vitro technologies where a very heterogeneous oocyte population, at different stages of growth and atresia, is used to produce embryos. It is possible that the presence of certain polymorphic genes that control apoptosis could be transferred from the oocyte to the resulting embryo, impairing its quality and its subsequent development (reviewed by ref. [29]). In fact, it has already been observed that maternal genotype has a strong effect on the incidence of cellular fragmentation in the embryo [30], probably mediated by early zygotic gene transcription. Moreover, cell survival/death regulatory molecules can be found in the stored pool of maternal mRNA in mice and human [31–33] and as a consequence they are likely to contribute in determining the fate of the embryo. Other factors that affect oocyte quality, such as cumulus cells, could also affect blastocyst yield [34]. Oocyte and cumulus cells are connected through gap junctions, which allow the interchange of molecules between these two cellular types (reviewed by ref. [35]). Apoptosis incidence in cumulus cells seems to affect oocyte quality and, as a consequence, subsequent embryo development [25–28].

Most of the molecular indicators for oocyte quality must be assessed by invasive methods, such as PCR or staining, so that the oocyte can no longer be used to produce embryos. As a consequence, it is necessary to establish a relationship between an invasive and a non-invasive quality marker, such as follicle diameter, COC morphology and oocyte diameter. Follicle diameter has been widely used as a selection parameter, and a relationship between follicle diameter and oocyte developmental competence has been established in many species (Cattle [36]; goat [37]; pig [38]; buffalo [39]). In cattle, follicles under 3 mm diameter contain oocytes with a diameter under 110 μm (reviewed by ref. [13]), which are considered to be developmentally incompetent oocytes [40,41], and follicles >6 mm are considered to suffer more incidence of apoptosis [42]. Another selection criterion is cumulus–oocyte complex (COC) morphology, despite the fact that contradictory results have been obtained when using this parameter as a marker of developmental competence: while many studies have found that COCs showing signs of early atresia give higher blastocyst rate [40,43–45], other studies obtained less [46] or the same developmental competence than COCs with no signs of atresia [21]. In what extent morphological features of apoptosis in COCs are related to real incidence of apoptosis in these complexes, and how the inductors of apoptosis present in cumulus cells could affect the oocyte developmental competence should be further studied. Although is not commonly used, oocyte diameter can also be used as a non-invasive selection parameter as it has been observed that blastocyst rate increases with oocyte diameter [47] (reviewed by ref. [13]). In cattle, cleavage and blastocyst rate is higher in oocytes with a diameter higher than 120 μm (reviewed by ref. [13]). Moreover, oocyte diameter has been related to incidence of apoptosis in COC [48] showing that an increasing level of atresia in COC was accompanied by a higher oocyte diameter.

The objective of the present study was to relate two non-invasive selection parameters (COC morphology and oocyte diameter) with embryonic development and apoptotic rate in COCs, oocytes and resulting blastocysts. For that purpose, embryonic development was assessed by cleavage and blastocyst rate, and apoptotic incidence was evaluated by an early (change in cytoplasmic membrane) and late (DNA fragmentation) marker of apoptosis.

## 2. Materials and methods

### 2.1. In vitro embryo production

Cow ovaries were obtained from a local abattoir and transported to the laboratory within 2 h. The ovaries were washed twice in physiological saline (0.9%) with 0.5% kanamycin (25 mg/ml, Gibco-BRL Life Technologies, Belgium) at 37 °C and COCs (cumulus–oocyte complexes) were recovered by aspiration from

follicles of 2–6 mm diameter. Only COCs with several layers of compact cumulus cells and homogeneous cytoplasm were selected for in vitro maturation (IVM). These immature COCs were cultured in 500 μl of modified bicarbonate buffered TCM-199 (Gibco-BRL Life Technologies, Belgium) supplemented with 20% heat-inactivated foetal calf serum (FCS) (Biochrom AG, Germany) in groups of 30–100 for 24 h at 39 °C and 5% $CO_2$ in humidified air.

Frozen-thawed bull semen was centrifuged on a Percoll gradient (45 and 90%; Pharmacia, Sweden) and washed. The sperm concentration was determined using a Bürker chamber. The sperm suspension was diluted in IVF-TALP medium (bicarbonate buffered Tyrode's medium supplemented with 6 mg BSA/ml) supplemented with 0.1 UI/ml heparin. Mature oocytes were washed in IVF medium and placed in groups of 100 in a four-well plate containing 250 μl of IVF medium in each well. Oocytes were cultured with 250 μl of the sperm suspension to obtain a final concentration of $1 \times 10^6$ spz/ml in the well. Oocytes and sperm were co-incubated at 39 °C in 5% $CO_2$ for 24 h.

At 24 h post-insemination (hpi), presumptive zygotes were denuded of the remaining cumulus cells and attached sperm cells, and washed. Groups of 25 embryos were placed in 50 μl droplets of SOF supplemented with 5% foetal calf serum, and covered with mineral oil. The embryos were incubated at 39 °C in 5% $CO_2$, 5% $O_2$ and 90% $N_2$ for 6 days. After 6 days of culture, the blastocysts obtained were analyzed for apoptosis.

### 2.2. Detection of apoptosis

#### 2.2.1. Annexin-V staining

The staining was performed with *Vybrant Apoptosis Assay kit #3* (Invitrogen, V13242). During the whole process, the reagents were kept at 37 °C to avoid false positives. Cells were stained with Annexin-V, a phospholipid binding protein that detects translocation of phosphatidyl-serine to the outer cytoplasmic membrane, which takes place during the early stages of apoptosis. At the same time, cells were stained with propidium iodide (PI), a membrane impermeable stain, to distinguish between live cells and dead cells. PI can only enter the cell when the cytoplasmic membrane has lost its integrity.

Briefly, samples were placed in 35 μl droplets containing 1× Annexin-V buffer, Annexin-V/FITC, PI and 1 mg/ml Hoechst 33342, and incubated for 15 min at 37 °C in the dark. After incubation cells were mounted on siliconized slides with vaseline bridges and observed under a fluorescent microscope.

Denuded oocytes were classified in three groups, representing (1) early apoptotic oocytes with intact Hoechst positive nuclei and a homogeneous annexin positive signal in the membrane (see Fig. 1A); (2) viable oocytes, with intact Hoechst positive nuclei and no annexin staining (see Fig. 1B); and (3) necrotic oocytes which showed PI positive red nuclei, which is indicative for membrane damage; in this last group, there were occasionally signs of annexin staining in the membrane. Oocytes showing a discontinuous green signal that came from the remaining membrane of cumulus cells projections [49] were also considered to be viable non-apoptotic oocytes (see Fig. 1C). While analyzing the oocytes, the progression of nuclear maturation was also recorded.

COCs were evaluated as a whole mount preparation, which made it impossible to count the number of cumulus cells positive or negative for annexin staining. We evaluated the distribution of the stained cells instead. The classification was the following: healthy COCs with no cells stained; early apoptotic COCs with cells only stained in green; necrotic COCs with cells only stained in red; and COCs with apoptotic (green signal) and necrotic cells (green and/or red signals). In this last group, we also considered if apoptotic or necrotic cumulus cells were the predominant in the whole COC (see Fig. 2A).

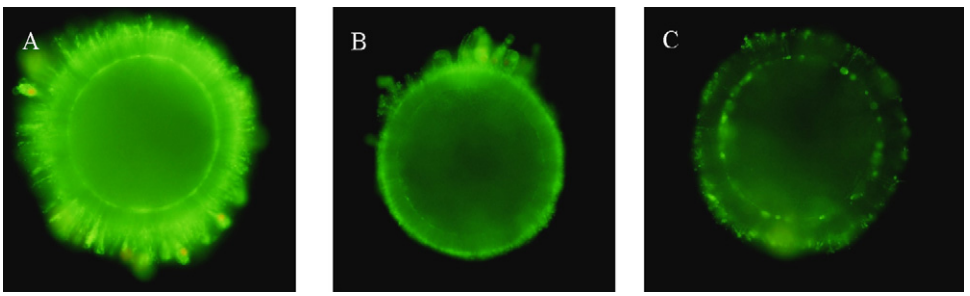


Fig. 1. Oocyte classification by Annexin-V staining: (A) Annexin-V positive (early apoptotic): a clear green signal is observed in the oocyte membrane. (B) Oocyte Annexin-V negative: no signal in the ooplasmic membrane. (C) Oocyte Annexin-V negative: green heterogenous signal in the ooplasmic membrane is considered to be a remnant of cumulus cell projections. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

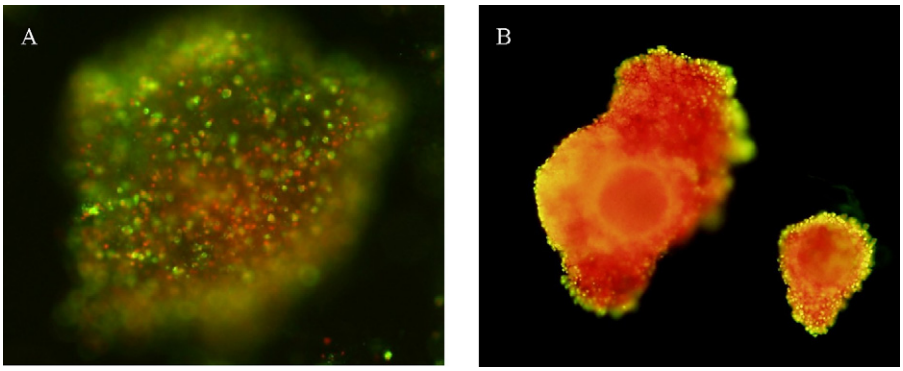


Fig. 2. (A) Annexin-V staining in COC and (B) COC with some TUNEL-positive cells in the outer cumulus layers.

Blastocyst cell number was determined by Hoechst staining, which let us to divide the blastocyst of each diameter group in three additional classes: blastocysts with less than 35 blastomeres; blastocysts containing 35–60 blastomeres; and blastocysts with more than 60 blastomeres. Classification of annexin-stained blastomeres was the same as used for oocytes (see Fig. 3). Results were represented as: EACR (early apoptotic cell ratio); LACR (late apoptotic cell ratio); and NCR (necrotic cell ratio).

After evaluation of oocytes and COCs, cells were washed four times in PVP (1 mg/ml polyvinyl-pyrrolidone in PBS), fixed in 4% paraformaldehyde overnight and used for TUNEL staining the day after. Blastocysts to be used for TUNEL assay were fixed in paraformaldehyde without performing Annexin-V staining first to avoid misinterpretation of the fluorescent signal.

### 2.2.2. *TUNEL assay*

Fixed samples were washed twice in 1 mg/ml PVP and incubated in 0.5% Triton X-100 (in PBS) for 1 h at room temperature. Afterwards, positive and negative control samples were incubated with 0.1 U/μl DNAse for 1 h at 37 °C. After DNAse treatment, the TUNEL staining was performed with *In Situ Cell Death Detection Kit* (Roche, 1684795) following the manufacturer's instruction. The negative control was incubated in the same conditions as the rest of the samples but without enzyme *Terminal deoxynucleotidyl transferase*. Subsequently, samples were treated with 50 μg RNAse/ml for 1 h at room temperature in the dark, and stained with 6.25 μg/ml propidium iodide for 15 min before mounting them on slides. Samples were evaluated by means of fluorescence microscopy.

Denuded oocytes were classified as: apoptotic, cells with fragmented nuclei (TUNEL-positive, green) (see Fig. 4A and B); and non-apoptotic, cells with intact nuclei (TUNEL-negative, red) (see Fig. 4A and C). At the same time, the nuclear maturation stage was recorded.

COCs were evaluated as a whole mount, and the COCs were classified depending on the distribution of the TUNEL-positive cells: viable, in COCs with no apoptotic cells; outer cumulus layers, when apoptotic

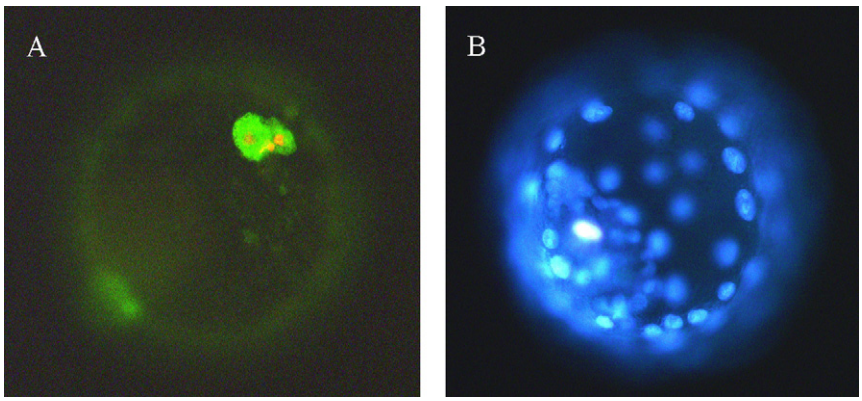


Fig. 3. Annexin-V staining in blastocysts: (A) Blastocyst with two apopotic and PI positive blastomeres and (B) the same blastocyst stained with Hoechst.

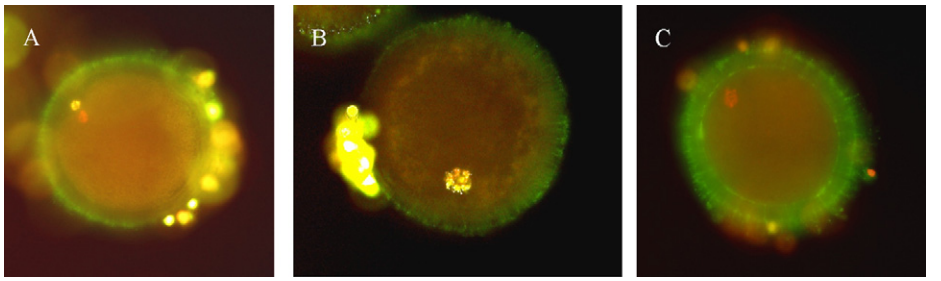


Fig. 4. TUNEL staining in oocytes: (A) MII-oocyte TUNEL-negative (red nucleus) with the polar body TUNEL-positive. (B) MI-oocyte TUNEL-positive (yellow nucleus). (C) MI-oocyte TUNEL-negative. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cells where only located in the outer layers (see Fig. 2B); and disperse, when apoptotic cumulus cells were distributed evenly throughout the COC.

In blastocysts, we determined the apoptotic cell ratio (ACRT, apoptotic cell ratio detected by TUNEL assay) as the number of TUNEL-positive blastomeres in relation to the total cell number of the blastocyst (see Fig. 5). Total cell number in blastocysts was determined, and each diameter group was divided in three additional classes as explained in annexin staining.

### 2.3. Experimental design

#### 2.3.1. Experiment 1: Relationship between oocyte diameter and prevalence of apoptosis in immature and mature oocytes

The objective of the first experiment was to evaluate if the degree of apoptosis in oocytes, before and after maturation, was different depending on their diameter. The oocyte diameter (without zona pellucida) was measured in all COCs before IVM and they were classified in three groups according to their size: Group A, $<$110 μm; Group B, 110–120 μm; Group C, $>$120 μm [74]. Half of the COCs of each diameter group were denuded in TCM-199 medium supplemented with 300 μg/ml hyaluronidase (Sigma, USA) at collection time to perform Annexin-V staining and TUNEL assay before maturation (0 h). The same oocytes used for Annexin-V staining were washed, fixed in paraformaldehyde 4% and stored at 4 °C until their use for TUNEL assay. The rest of COCs were placed in IVM droplets for 24 h, and after that period the matured COCs were denuded to perform Annexin-V staining and TUNEL assay after maturation (24 h).

#### 2.3.2. Experiment 2: Relationship between oocyte diameter and prevalence of apoptosis in immature and mature cumulus–oocyte complexes

The objective of the second experiment was to evaluate if the degree of apoptosis in the COCs, before and after maturation, was depending on the oocyte diameter. The oocyte diameter was measured and oocytes were classified in the same groups as specified in the first experiment.

Each COC group was used to perform Annexin-V staining and TUNEL staining at 0 h (before maturation) and 24 h (after maturation).

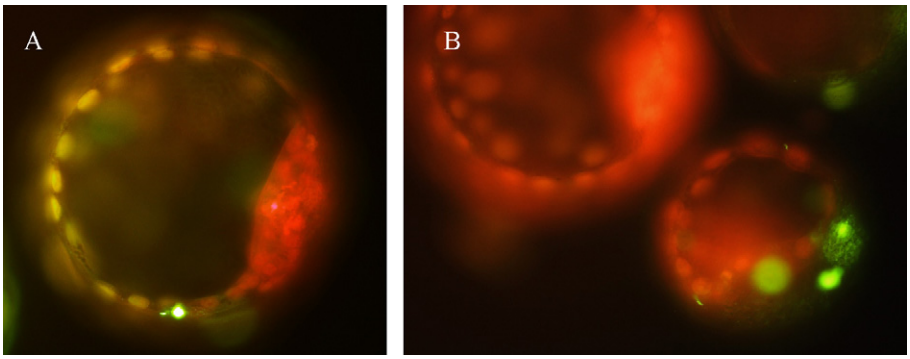


Fig. 5. TUNEL assay in blastocysts: (A) Blastocyst with one positive cell (in yellow); (B) Blastocyst with no apoptotic cells (up) and blastocyst with three TUNEL-positive cells (down). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 2.3.3. Experiment 3: Relationship between oocyte diameter and prevalence of apoptosis in blastocysts derived from differently sized oocytes

The objective of the third experiment was to study the blastocyst rate and the degree of apoptosis in blastocysts obtained from oocytes of different size. Again, the diameters groups were the same as described in experiments 1 and 2, but the size was measured before in vitro culture (IVC) in denuded zygotes.

Cleavage rates were determined at 48 hpi for each group. At 7 days post-insemination (dpi) half of the blastocyst of each group were analyzed by annexin staining, and the other half were fixed in paraformaldehyde 4% overnight and used for TUNEL assay the day after.

### 2.4. Statistical analysis

Logistic regression analyses, including the effect of replicate, were performed to compare the proportion of TUNEL and Annexin-V positive cells in the different groups of oocytes and COC, and to assess the developmental competence among diameter groups. The apoptotic cell ratio in blastocysts, detected by Annexin-V staining and TUNEL assay in different diameter groups, was analyzed using analyses of variance, also including the effect of replicate. Differences were considered significant when $P < 0.05$. All analyses were performed using SPSS 12 for Windows (Analytical Software, Chicago, IL).

## 3. Results

### 3.1. Experiment 1: Relationship between oocyte diameter and prevalence of apoptosis in immature and mature oocytes

The results of Annexin-V and TUNEL staining in bovine oocytes are represented in Table 1. When Annexin-V staining was performed, we could not detect any statistical differences in the rate of early apoptotic oocytes in the three oocyte groups either before or after maturation. Less apoptosis was observed in mature than in immature oocytes in Groups B and C when annexin staining was used. Oocyte nuclear stage had no effect on the outcome of Annexin-V staining.

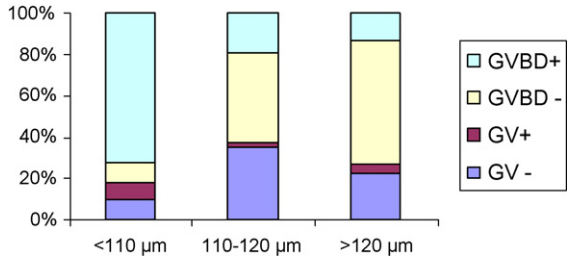


Fig. 6. Distribution of nuclear stage and TUNEL outcome depending on oocyte diameter before maturation. GV+ or GVBD+: oocytes at GV or GVBD nuclear stage, respectively, that were positive for TUNEL staining. GV− or GVBD−: viable oocytes at GV or GVBD nuclear stage, respectively.

When late apoptotic events were assessed, we observed that the percentage of TUNEL-positive oocytes was statistically higher in immature oocytes with a diameter below 110 μm in comparison with larger oocytes. The apoptotic rate decreased significantly during maturation, with only 10.5% or less of mature oocytes showing signs of late apoptosis and no statistical differences in rate of apoptotic oocytes between groups.

The relationship between nuclear stage and late apoptotic events is represented in Figs. 6 and 7. Most of the TUNEL-positive immature oocytes were at germinal vesicle breakdown stage (GVBD), and this proportion was statistically higher in oocytes with a diameter below 110 μm. In mature oocytes the nuclear stage had no effect on the TUNEL staining outcome, and we observed that the proportion of oocytes reaching metaphase II stage increased with oocyte diameter.

Table 1
The incidence of early and late apoptosis, as assessed by Annexin-V and TUNEL staining in immature and in vitro matured bovine oocytes grouped according to oocyte diameter

| Oocyte diameter | <110 μm | | | 110–120 μm | | | >120 μm | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | Apoptotic | % | Total | Apoptotic | % | Total | Apoptotic | % |
| Immature Annexin | 23 | 5 | 21.7 | 53 | 19 | $35.9^{1}$ | 64 | $13^{*}$ | $18.8^{1}$ |
| Immature TUNEL | 11 | 9 | $81.8^{a1}$ | 53 | 11 | $20.8^{b1}$ | 53 | 9 | $17.0^{b}$ |
| Mature Annexin | 15 | 2 | 13.3 | 102 | 5 | $4.9^{2}$ | 81 | 2 | $2.5^{2}$ |
| Mature TUNEL | 23 | 2 | $8.7^{2}$ | 152 | 11 | $7.2^{2}$ | 124 | 13 | 10.5 |

Values in the same row with different superscripts (a,b) differ significantly ($P < 0.05$). Values in the same column and assay with different numbers (1,2) differ significantly ($P < 0.05$).

* One oocyte stained with PI, and thus it can also be considered as necrotic.

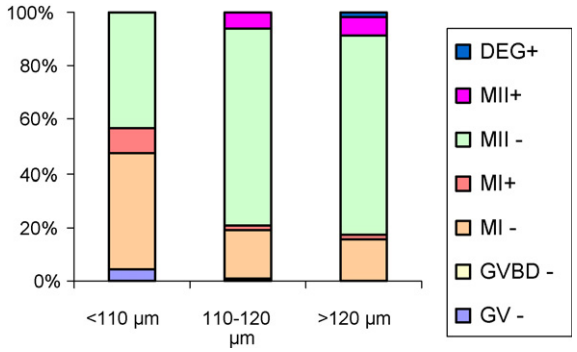


Fig. 7. Distribution of nuclear stage and TUNEL outcome depending on oocyte diameter after maturation. MI+ or MII++: oocytes at MI or MII nuclear stage, respectively, that were positive for TUNEL staining. DEG+: degenerate oocytes that were positive for TUNEL staining. GV− or GVBD−: viable oocytes at GV or GVBD nuclear stage, respectively.

### 3.2. Experiment 2: Relationship between oocyte diameter and prevalence of apoptosis in immature and mature cumulus–oocyte complexes

The aim of the second experiment was to study if the apoptosis degree of cumulus–oocytes complexes was related to oocyte diameter.

When Annexin-V staining was performed, we did not detect any difference among diameter groups before maturation. It is important to notice that we observed positive cells in the COCs of the three groups before and after maturation. After maturation, a higher percentage of COCs with early apoptotic cells was detected in Group A in comparison to the other two groups. At the same time, after maturation the proportion of COCs with late apoptotic and/or necrotic cumulus cells was significantly higher in Groups B and C (Table 2).

When TUNEL assay was performed in immature COCs, we observed that the percentage of COCs with apoptotic cells was statistically lower when the oocyte diameter was 110–120 μm in comparison to the other groups. Apoptotic cumulus cells were located exclusively in the outer layers (Table 3).

During IVM, there was a significant increase in the rate of COCs with apoptotic cells in Group B. We could also observe that the distribution of the apoptotic cells in the COCs varied during IVM: before IVM they were almost only located in the outer cumulus cells (see Fig. 2B), while after IVM, we observed a significant increase in positive cells distributed in a dispersed way in the COC in the two biggest groups (Table 3).

After maturation, there were no differences detected among diameter groups. The percentage of COCs showing no apoptotic cumulus cells was higher in the largest group, but it was not statistically different (Table 3).

### 3.3. Experiment 3: Relationship between oocyte diameter and prevalence of apoptosis in blastocysts derived from differently sized oocytes

Cleavage rate in embryos originating from oocytes with the smallest diameter was 50%, which was significantly lower than for embryos in the other groups (Table 4). Furthermore, blastocyst rate was significantly higher in Groups B and C when compared to the smallest group (<110 μm: 9.18%; 110–120: 21.20%; >120: 23.97%) (Table 4). Relation blastocyst rate/cleavage was also higher in the embryos coming from oocytes with the largest diameters (18.36% in Group A versus 36.63% and 40.81% in Groups B and C, respectively) (Table 4). Moreover, none of the blastocysts in Group A were hatched at 7 dpi.

Blastocysts from the Group C showed significantly less late apoptotic and necrotic cells than Group B when annexin staining was performed (late apoptotic: Group B, 3.75%; Group C, 1.57%; Necrotic: Group B, 1.33%; Group C, 0.22%) (Table 5). We detected no differences in ACRT among diameter groups when TUNEL assay was performed (Group A, 12.37%; Group B, 12.28%; Group C, 12.54%).

We also evaluated the apoptosis rate among the different groups depending on blastocyst cell number (Table 6). We detected significant differences among

Table 2
Annexin staining in COCs before and after maturation depending on oocyte diameter

| Annexin staining | <110 μm | | | | 110–120 μm | | | | >120 μm | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | Viable | EA (%) | LA/Necrotic (%) | *N* | Viable | EA (%) | LA/Necrotic (%) | *N* | Viable | EA (%) | LA/Necrotic (%) |
| Immature COC | 15 | 0 | 0 | 15 (100) | 40 | 0 | 2 (5) | 38 (95) | 43 | 0 | 3 (6.98) | 40 (93.02) |
| Mature COC | 15 | 0 | $3^{a}$ (20) | $12^{\alpha}$ (80) | 61 | 0 | $2^{b}$ (3.28) | $59^{\beta}$ (96.72) | 60 | 0 | $0^{b}$ | $60^{\beta}$ (100) |

Values in the same row and different diameter and classification group, with different superscripts (a,b or α,β) differ significantly ($P < 0.05$). *N*, total complexes evaluated; EA, early atretic; LA, late atretic.

Table 3
TUNEL assay in COCs before and after maturation depending to oocyte diameter

| TUNEL assay | <110 μm | | | | 110–120 μm | | | | >120 μm | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Viable (%) | Distribution of apoptotic cells (%) | | Total | Viable (%) | Distribution of apoptotic cells (%) | | Total | Viable (%) | Distribution of apoptotic cells (%) | |
| | | | Outer cumulus layers | Dispersed | | | Outer cumulus layers | Dispersed | | | Outer cumulus layers | Dispersed |
| Immature COC | 16 | $0^{a}$ | $16^{\alpha 1}$ (100) | 0 | 58 | $24^{b1}$ (41.38) | $34^{\beta}$ (58.62) | $0^{1}$ | 61 | $12^{a}$ (19.67) | $49^{\alpha 1}$ (80.33) | $0^{1}$ |
| Mature COC | 23 | 1 (4.35) | $16^{2}$ (69.56) | 6 (26.10) | 65 | $4^{2}$ (6.15) | 43 (66.15) | $18^{2}$ (27.70) | 69 | 12 (17.39) | $41^{2}$ (59.42) | $16^{2}$ (23.19) |

Values in the same row and different diameter and classification groups with different superscripts differ significantly (a,b or α,β). Values in the same column with different superscripts (1,2) differ significantly ($p > 0.05$).

groups in the necrotic cell ratio when blastocysts had more than 35 blastomeres. The ACRT in blastocysts with more than 60 cells was significantly lower in the largest oocyte group compared to Group B. We observed that the early apoptotic cell ratio and ACRT in blastocysts within the same diameter group decreased with increasing cell numbers.

## 4. Discussion

Oocyte quality is the most important parameter that will determine blastocyst rate (reviewed by ref. [9]). Generally, COC morphology is used as a selection parameter of oocyte quality because a relationship between bovine oocyte morphology and developmental competence has been established [50]. In addition to COC morphology, oocyte diameter can also be used as a non-invasive parameter, since a relationship between oocyte diameter and its developmental competence has been shown [39,51]. In this study, we combined two non-invasive selection parameters in order to select for oocytes of different quality and to assess possible effects on blastocyst rate and quality in terms of incidence of apoptosis.

In this study, apoptosis assessed by Annexin-V staining and TUNEL assay was detected in both immature and mature oocytes. Several studies on the incidence of apoptosis in bovine oocytes show contradictory results: Yuan et al. [25] detected no apoptotic oocytes before and after maturation, while Warzych et al. [52] and Matwee et al. [53] observed apoptosis both in mature (11.2 and 23%, respectively) and immature oocytes (1.4 and 7%, respectively). Moreover, apoptosis has also been observed in ovulated murine oocytes [49,54], ovulated human oocytes [49] and in vitro-matured human oocytes [55]. In our study, apoptosis detected in immature oocytes by both Annexin-V and TUNEL staining was higher than in the studies reported previously. Maturation reduced the occurrence of apoptosis in all groups of oocytes analyzed, with similar results for mature oocytes than those from Warzych et al. [52]. Although a negative effect of culture in vitro in the incidence of apoptosis in oocyes has been reported [49,56,57], our results do not show a negative influence of in vitro maturation on oocyte quality in terms of apoptosis, which have also been observed by Van Blerkom and Davis [49], who reported a reduction of apoptotic incidence in oocytes cultured for 4 days. Discrepancies among authors about the effect of in vitro maturation on the incidence of apoptosis could be due to differences in media composition [52]. However, we cannot exclude the

Table 4
Embryos developing from oocytes with different diameters

| Oocyte diameter | *N* | Cleavage at 4 dpi (%) | Hatched blastocysts at 7 dpi (%) | Total blastocyst rate (%) | Blastocyst/cleavage (%) |
|---|---|---|---|---|---|
| <110 μm | 294 | 147 (50)[a] | 0 | 27 (9.18)[a] | 18.36[a] |
| 110–120 μm | 1047 | 606 (57.88)[b] | 3 (0.29) | 222 (21.20)[b] | 36.63[b] |
| >120 μm | 584 | 343 (58.73)[b] | 4 (0.68) | 140 (23.97)[b] | 40.81[b] |

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

Table 5
Detection of apoptosis in day 6 blastocysts from oocytes of different diameter

| Oocyte, Ø (μm) | Annexin-V staining | | | | TUNEL assay | |
|---|---|---|---|---|---|---|
| | Blastocysts evaluated | EACR (mean ± S.D.) | LACR (mean ± S.D.) | NCR (mean ± S.D.) | Blastocysts evaluated | Apoptotic cell ratio (mean ± S.D.) |
| <110 μm | 13 | 6.49 ± 3.84 | 3.36 ± 6.34[a,b] | 1.65 ± 2.82[a,b] | 11 | 12.37 ± 7.23 |
| 110–120 μm | 75 | 9.74 ± 8.00 | 3.75 ± 5.66[a] | 1.33 ± 2.37[a] | 67 | 12.28 ± 16.35 |
| >120 μm | 45 | 8.56 ± 7.87 | 1.57 ± 2.61[b] | 0.22 ± 0.86[b] | 56 | 12.54 ± 8.59 |

Values in the same column with different superscripts (a,b) differ significantly ($P < 0.05$). EACR, early apoptotic cell ratio; LACR, late apoptotic cell ratio; NCR, necrotic cell ratio.

possibility that manipulation may have induced an early stage of atresia in our immature oocytes, which would be reverted by our in vitro culture conditions.

Differences in the incidence of apoptosis in oocytes among the diameter groups studied was only observed in immature oocytes, and the smallest oocytes showed the highest rates of late apoptosis. After in vitro maturation, no differences were detected among groups. These results could indicate: (1) the quality of the smallest oocytes, as assessed by apoptosis detection, is lower than the largest oocytes, which would be reflected in the low blastocyst yield obtained from these oocytes; or (2) the smallest oocytes have not yet finished their growth period, and may be they are more sensitive than the largest oocytes to manipulation.

We detected an effect of nuclear stage on the incidence of late apoptotic events in immature oocytes: most of the apoptotic oocytes had already resumed meiosis (<110 μm: 88.88%; 110–120 μm: 90.91%; >120 μm: 77.77%), while the proportion of oocytes at

Table 6
Detection of apoptosis in day 6 blastocysts from oocytes of different diameter depending on blastocyst cell number

| Oocyte, Ø | Annexin-V staining | | | | TUNEL assay | |
|---|---|---|---|---|---|---|
| | Blastocysts evaluated | Early apoptotic cell ratio (mean ± S.D.) | Late apoptotic cell ratio (mean ± S.D.) | Necrotic cell ratio (mean ± S.D.) | Blastocysts evaluated | Apoptotic cell ratio (mean ± S.D.) |
| <35 cells | | | | | | |
| <110 μm | 0 | 0 | 0 | 0 | 0 | |
| 110–120 μm | 9 | 19.95 ± 6.31[1] | 8.86 ± 9.13[1] | 2.01 ± 4.00 | 8 | 37.34 ± 31.10[1] |
| >120 μm | 7 | 18.79 ± 8.38[2] | 2.99 ± 4.54 | 0.71 ± 1.88 | 6 | 19.68 ± 14.58[1] |
| 35–60 cells | | | | | | |
| <110 μm | 7 | 7.09 ± 3.60 | 5.09 ± 8.45 | 2.45 ± 3.62[a] | 4 | 19.68 ± 5.53[1] |
| 110–120 μm | 31 | 11.40 ± 8.11[2] | 4.34 ± 5.93[1,2] | 1.43 ± 2.35[a,b] | 12 | 16.27 ± 16.99[2] |
| >120 μm | 13 | 10.90 ± 8.65[1] | 1.92 ± 2.67 | 0.18 ± 0.66[b] | 11 | 14.62 ± 9.38[1,2] |
| >60 cells | | | | | | |
| <110 μm | 6 | 5.78 ± 4.33 | 1.34 ± 1.26 | 0.72 ± 1.21[a,b] | 7 | 8.20 ± 3.99[a,b2] |
| 110–120 μm | 35 | 5.65 ± 4.95[3] | 1.91 ± 2.89[2] | 1.07 ± 1.83[a] | 47 | 7.00 ± 5.04[a2] |
| >120 μm | 25 | 4.49 ± 2.81[2] | 1.00 ± 1.67 | 0.11 ± 0.40[b] | 39 | 10.68 ± 6.60[b2] |

Values in the same column and blastocyst cell class (<35 cells, 35–60 cells or >60 cells) and with different superscripts (a,b) differ significantly ($P < 0.05$). Values in the same column and diameter group (<110 μm, 110–120 μm, >120 μm) and with different superscripts (1,2) differ significantly ($P < 0.05$).

GVBD stage decreased in the non-apoptotic ones (<110 μm: 50%; 110–120 μm: 57.14%; >120 μm: 72.72%). No immature oocyte beyond GVBD was detected. These results would be in accordance with Warzych et al [52] who detected that the apoptotic rate was higher in ''immature'' oocytes that had reached MI or MII (50%), than those that still were at GV (0.70%). It has been suggested that apoptosis is a mechanism to eliminate oocytes that have resumed meiosis inside the follicle. Therefore, apoptosis in our immature oocytes could be a signal that they come from follicles that could not maintain meiotic arrest in those oocytes. In which way this premature meiotic resumption could affect blastocyst rate should be further studied, although it has been suggested that oocytes showing early signs of atresia suffer a phenomenon similar to maturation [58], which confer higher embryonic developmental competence [40]. de Wit et al. [45] also found that COC showing morphological characteristics of early stage of atresia seemed to mature in vitro faster than healthy COCs, because of a premature oocyte-cumulus uncoupling.

Several studies have concluded that the detection of the incidence of apoptosis in COCs could be a good indicator of oocyte developmental competence [46,59,60]. Cumulus cells and oocytes communicate in a bidirectional way through gap junctions [61]. Cumulus cells play an important role in maintaining oocyte meiotic arrest, support oocyte cytoplasmic maturation and participate in the induction of meiotic resumption (reviewed by ref. [35]). In addition, presence of cumulus cells during in vitro maturation is essential to improve blastocyst rate [34], maybe due to the protective effect of the cumulus cells against oxidative stress that could induce apoptosis in the oocytes [62]. Our second experiment tried to establish if differences in blastocyst rate among oocyte diameters could be related to the incidence of apoptosis in the corresponding cumulus cells. At the time of collection, we observed that most of the apoptotic cumulus cells were located in the outer layers, which is in accordance with the results of Yuan et al. [46]. The absence of apoptotic cumulus cells in the corona of the COC is explained by the anti-apoptotic effect of a gradient of paracrine factors secreted by the oocyte, probably BMP15 (Bone morphogenetic protein-15) and BMP6 [63]. A differential distribution of the late apoptotic cumulus cells detected by TUNEL before and after IVM was observed, indicating that they distribute in a more dispersed way when in vitro maturation progresses. Moreover, after maturation there was an increase in the percentage of COCs showing late apoptotic cumulus cells in Group B. Yuan et al. [46] and Ikeda et al. [64] obtained similar results and they detected an increase of apoptotic cumulus cells after maturation, detected by TUNEL and ligation mediated-PCR, respectively.

Annexin-V assay in COCs revealed that, after maturation, the percentage of COCs showing late apoptotic cells was higher in Groups B and C than in Group A, which had more COCs with early apoptotic cumulus cells. These results could also indicate a deficiency in cumulus expansion and maturation in COCs containing the smallest oocytes. In fact, it was also observed that only 43.47% of the smallest oocytes were able to reach metaphase II after in vitro maturation, while the percentage of MII oocytes increased up to 78.94 and 81.45% in Groups B and C, respectively.

Apoptosis incidence is related to embryo quality [65]. Apoptosis plays a very important role during pre- and post-implantation development, removing abnormal cells or cells that are no longer required and controlling the embryo cell number. The protective role of apoptosis in the pre-implantation period can turn into damage if the incidence in the embryo is very high. However, a detrimental threshold of apoptosis incidence has not been established yet, and therefore consequences of apoptotic incidence in blastocysts are difficult to assess. Our results of apoptosis rate in bovine blastocysts show no differences in ACR among diameter groups when TUNEL assay was performed (average about 12%). Other studies using TUNEL assay in in vitro produced blastocysts showed an average apotic index of either 10.3% [66], 4–9% [67], 12% [68] and 9.8–14.2% [69], which is very similar to our results. In addition to TUNEL assay, Annexin-V staining was also performed to analyze apoptosis in blastocysts, and we observed a reduction of late apoptotic cell ratio and necrotic cell ratio in blastocysts of Group C. Apoptosis incidence, although lower than in blastocysts produced in vitro, has been also observed in in vivo bovine, porcine, rodent [67], primate and human blastocysts [70]. Therefore, it is difficult to determine in what extent the apoptosis detected in our blastocysts can affect their quality. However, as we did not detect any difference in developmental competence among Groups B and C, we can establish that the differential incidence of late stage of apoptosis and necrosis detected in blastocysts derived from each diameter group seems to have no effect on the blastocyst rate.

The incidence of apoptosis is variable during embryo development: it has been reported that it appears after compaction in human embryos, and varies during progression of blastocyst stage [71], while in murine blastocysts there is a wave of death cell during blastocyst expansion and minimal levels of apoptosis incidence are

found in the late blastocyst stage, before implantation [67,72]. We separated our blastocysts in three additional groups depending on their cell number to study if apoptosis in bovine blastocysts varies depending on the stage too. In most cases, we observed a decrease in ACR as the cell number increased, both with Annexin-V and TUNEL assay, in all diameter groups. This decrease in apoptosis incidence is explained by the increase of blastocyst cell number rather than a reduction of the total apoptotic blastomeres in the blastocyst.

Our results of developmental competence indicate that cleavage and blastocyst rate increased with oocyte diameter, as shown in previous studies [39,51]. Considering our results on the incidence of apoptosis in oocytes, cumulus cells and blastocysts, it is difficult to find a clear relationship between the parameters analyzed and developmental competence of the oocyte. Although we found a higher apoptosis rate in the smallest oocytes at the time of collection time, the reduction found after maturation may indicate that it is not the main cause of the reduced developmental competence of these oocytes. On the other hand, we observed more apoptosis in cumulus cells of the smallest oocytes at collection time, which could be indicative for the low developmental potential of these oocytes. However, it is difficult to affirm that it is the only cause of the reduced blastocyst rate achieved in these oocytes. It is accepted that small oocytes have less developmental competence due to an insufficient cytoplasmic maturation, and they normally do not reach the blastocyst stage because of a lack of accumulation of RNA and proteins, necessary to maintain embryonic development beyond the embryonic genome activation [12], which takes place in bovine embryos at the 8–16 cell stage [73]. Therefore, it seems more probable that the reduced developmental competence of the smallest oocytes is due to the fact that they have not yet finished their growth rather than a higher incidence of apoptosis.

In conclusion, oocyte selection by COC morphology is not enough to select only non-apoptotic COCs. The use of oocyte diameter as an additional selection parameter helps to select the most competent oocytes, but this developmental competence is not related to a difference in incidence of apoptosis among oocyte diameters.

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# EFFECT OF PREVALENCE OF APOPTOSIS IN OOCYTES AND CUMULUS CELLS, ASSESSED BY TUNEL ASSAY, ON EMBRYO DEVELOPMENT IN PREPUBERTAL GOATS

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**ABSTRACT**

Oocyte population recovered from prepubertal goats is very heterogeneous, and strict selection criteria are needed to recover the healthiest and most competent oocytes. The aim was to study apoptosis, assessed by TUNEL assay, in prepubertal goat COCs from different diameters and morphology, and its effect in embryo development. Oocytes were divided in three categories depending on the diameter: A: 110-125 µm, B: 125-135 µm and C: >135 µm. Each diameter category was classified in two groups depending on COC morphology: Healthy (H: compact cumulus layers and homogeneous cytoplasm) or Early atretic (EA: granulated cytoplasm and/or dispersed cumulus layers). TUNEL assay was performed in COCs before and after maturation, and in blastocysts. Before maturation, apoptosis in oocytes and cumulus cells (CCs) was related to oocyte diameter, only in H group (oocytes, A: 51. 6 %, B: 43.3 %, C: 12.1 %; CCs, A: 91.5 %, B: 65.8 %, C: 64.9 %). After IVM, the oocyte apoptosis decreased in the largest oocytes, in both morphology groups; on the contrary, the apoptosis increased in CCs. The blastocyst rate increased with increasing oocyte diameter, and it was higher in H than in EA oocytes (HA: 0%, HB: 5.3 %, HC: 14.4 %; EA A: 0.3 %, EA B: 4.1 %, EA C: 5.1 %). COCs diameter and morphology had no effect on the percentage of apoptosis in blastocysts cells. In conclusion oocyte developmental competence in prepubertal goats is influenced by oocyte diameter and COC morphology. Apoptosis in oocytes and cumulus cells could affect embryo development, but not prevalence of apoptotic blastocyst cells.

Keywords: apoptosis, oocyte, goat, TUNEL assay

## 1. INTRODUCTION

Apoptosis is a type of programmed cellular death that allows the regulation of biological processes, such as morphogenesis and tissular homeostasis, through the removing of abnormal cells or cells that are no longer required (Steller, 1995). In the ovary, apoptosis is the responsible of follicular atresia, by which most of the follicles present at birth are lost during further development. Follicular atresia can occur at any stage of follicular development, but the follicular compartment where atresia initiates differs: in antral follicles, atresia is initiated with degeneration of granulosa cells (Irving-Rodgers et al., 2001) and the oocyte is only affected during the last stage of follicular atresia (Driancourt et al., 1991; Yang and Rajamahendran, 2000; Yang and Rajamahendran, 2002); however, in preantral follicles, cell death is often initially observed within the oocyte (Marion et al., 1968; Reynaud and Driancourt, 2000). Not only the compartment initially affected, but also the mechanism of granulosa cell death varies depending on follicle size (Alonso-Pozos et al., 2003). The process of atresia occurs continuously from birth until the complete depletion of the follicle pool (Hirshfield, 1991; Hsueh et al., 1994; Kaipia and Hsueh, 1997), and more than half of follicles present in an ovary in a certain moment are undergoing atresia at different stages (Kruip and Dieleman, 1982). This is especially important when slaughtered female ovaries are used as source of oocytes for in vitro embryo production, which means that most of these oocytes may come from atretic follicles and, therefore, blastocyst production might be impaired.

Many approaches have tried to find out the parameters that confer oocyte developmental competence, such as stage of the estrous cycle (Hagemann et al., 1999), hormonal patterns (Kruip and Dieleman, 1982), composition of follicular fluid (Anifandis et al., 2005; Chang et al., 2002; Chiu et al., 2002; Wunder et al., 2005), follicular diameter (Blondin and Sirard, 1995; Crozet et al., 1995; Hagemann et al., 1999; Lonergan et al., 1994) and follicular atresia (Blondin and Sirard, 1995). However, the recovery of oocytes

from slaughtered female ovaries for in vitro embryo production makes difficult the use of some of those parameters to select the most competent oocytes. In addition, when prepubertal goats are used as oocyte donors, oocyte recovery must be done by slicing the ovarian surface, and not by follicular aspiration as in adults, which let us the obtaining of a high number of oocytes but without considering diameter of follicles or their degree of atresia. Consequently, the oocyte population recovered from prepubertal goats is very heterogeneous, and therefore a strict selection must be done in order to select the most competent oocytes.

Cumulus-oocyte complex (COC) morphology is a good selection parameter since it is related to the grade of atresia of the follicle that comprises it (Blondin and Sirard, 1995; de Wit et al., 2000). The strong relationship between cumulus cells and the oocyte through gap junctions allows the interchange of molecules in a bidirectional way (Tanghe et al., 2002); therefore, any factor that could affect cumulus cells, such as apoptosis, can also be reflected in a lower oocyte quality and, as a consequence, in vitro embryo production can be impaired. In general, it is accepted that COCs that come from non-atretic follicles present a compact cumulus cell layers and homogeneous oocyte cytoplasm (de Wit et al., 2000) and have higher developmental competence (Corn et al., 2005; Lee et al., 2001; Yuan et al., 2005; Zeuner et al., 2003). However, contradictory results have been obtained in other studies, concluding that COCs showing mild signs of atresia yield higher blastocyst rate (Blondin and Sirard, 1995; Boni et al., 2002; de Wit et al., 2000; Hazeleger and Stubbings, 1992).

The study of apoptosis in oocytes can be also a good marker of oocyte quality and its capability to develop into a viable embryo. Moreover, some evidence support the idea that apoptosis in oocyte can also affect embryo quality, due to the presence of molecules that regulate the apoptotic mechanism in the stored maternal mRNA in the oocyte (Exley et al., 1999; Jurisicova et al., 1998; Metcalfe et al., 2004) which is used to maintain oocyte maturation, fertilization and embryo development until the embryonic

genome activation (Bachvarova, 1992; Gandolfi and Gandolfi, 2001), or the strong effect that maternal genotype have in the cellular fragmentation of the embryo (Han et al., 2005). Apoptosis in blastocyst is a feature of normal development (Hardy, 1997); however, high incidence of apoptosis in blastocyst results in a compromise of embryonic development and may lead to formation of abnormalities in the fetus (Brison and Schultz, 1997). Therefore, apoptosis in the oocyte can be reflected not only in a lower developmental competence, but in a decrease of embryo quality assessed in terms of incidence of apoptosis in blastocysts obtained. However, few approaches have been made in order to find out characteristics in the oocyte related to apoptosis, and only oocyte diameter has been related to level of atresia in the COC (de Wit and Kruip, 2001).

TUNEL assay was developed by Gavrieli et al. (1992). This technique allows the identification of apoptotic cells by the use of an enzyme, the terminal deoxynucleotidil transferase (Tdt), which catalizes the addition of labelled dUTPs to the fragmented DNA. This method allows the analysis of individual cells, so that apoptotic cells can be located and its percentage quantified. Many authors have used TUNEL assay in apoptosis detection in oocytes and embryos from many species (bovine: Warzych et al., 2007; Yuan et al., 2005; Zeuner et al., 2003; murine: Brison and Schultz, 1997; human: Corn et al., 2005; Jurisicova et al., 1998; porcine: Kidson et al., 2004).

The aim of the present study was to evaluate the influence of apoptosis in oocytes and cumulus cells, assessed by TUNEL assay, in oocyte capability to reach blastocyst stage and blastocyst quality. In addition, the use of COC morphology and oocyte diameter as parameters to select the most competent oocytes was also evaluated.

## 2. MATERIAL AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

### *2.1. Oocyte collection and IVM*

Ovaries from prepubertal goats, approximately 2 months old, were recovered from a local slaughterhouse and transported within 2 hours to the laboratory at 38.5 ºC in PBS (Dulbecco's phosphate-buffered saline, P-4417) with 50 µg/mL gentamycin (G-3632). Ovaries were washed three times and COCs (cumulus-oocyte complexes) were recovered by slicing in TCM199 (M-2520) supplemented with 2.2 mg/mL $NaHCO_3$, 2% (v/v) steer serum (Donor Bovine Serum? , CanSera, Canada) and 50 ?g/mL gentamycin.

COCs were matured in TCM199 medium (M-7528) supplemented with 275 ?g/mL sodium pyruvate (P-3662), 146 µg/mL L-glutamine (G-5763), 10% (v/v) steer serum, 10 µg/mL o-LH (L-5269), 10 µg/mL o-FSH (F-4520), 1 µg/mL 17-ß estradiol (E-2257), 400 µM cysteamine (M-9768) and 50 µg/mL gentamycin (G-3632). Groups of 20-25 COCs were transferred to 100 µL microdrops of maturation medium under mineral oil (M-3516) and incubated for 27 hours at 38.5 ºC in a humidified air atmosphere with 5% $CO_2$.

### *2.2. Sperm capacitation and IVF*

Fresh semen was collected from two males of proven fertility and transported to the laboratory at 38.5 ºC within 30 minutes. Motility of the ejaculates was evaluated under a stereomicroscope. Motile sperm fraction was separated by the swim-up method (Parrish et al., 1986), whereby 70 µL of sperm were layered below 2 mL of mDM (Defined medium, Brackett and Oliphant, 1975; modified by Younis et al., 1991), and incubated for 45 min at 38.5 ºC in a humidified air atmosphere with 5% $CO_2$. After incubation, the supernatant was recovered and centrifuged at 170 x g for 3 minutes. The pellet was resuspended in mDM (1:1; v/v) containing heparin (H-3149) and

ionomycin (I-0634) (Final concentration: 10 µg/mL heparin and 200 nM of ionomycin) and incubated for 15 min in a humidified air atmosphere with 5%$CO_2$ and 38.5 ºC (Wang et al., 2002). Sperm concentration was determined using a Neubauer chamber cell.

After maturation, groups of 20 to 25 oocytes were transferred to 100 µL microdrops of modified Tyrode's medium (TALP) as described by Parrish et al. (1986), supplemented with 1 µg/mL hypotaurine (H-1384) and 0.3 mg/mL glutathione (G-6013) under mineral oil. Oocytes were co-cultured with capacitated spermatozoa in a final concentration in the microdrop of 4 x $10^6$ sperm cells/mL.

*2.3. In vitro embryo culture*

At 24 hours post-insemination, presumptive zygotes were washed to remove both remnant cumulus cells and attached sperm cells. Groups of 18-25 embryos were placed in 50 µl droplets of SOF medium (Synthetic Oviductal Fluid, Holm et al., 1999) and covered with mineral oil. The embryos were incubated at 38.5°C in 5% $CO_2$, 5% $O_2$ and 90 % $N_2$ for 7 days. At 48 hpi, 0.1 µL fetal bovine serum (F-7524) per embryo was added to the medium. At the end of the culture period (8 days post-insemination), total cell number of embryos was evaluated by Hoechst 33342 staining (B-2261) under a fluorescence microscope.

*2.4. TUNEL Assay (Tdt-mediated dUTP nick-end labelling)*

Samples were fixed in 4% paraformaldehyde overnight before performing TUNEL assay. Fixed samples were washed twice in 1 mg/mL PVP and incubated in 0.5% Triton X-100 (in PBS) for 1 hour at room temperature. Afterwards, positive and negative control samples were incubated with 0.1 U/ µL DNAse for 1 hour at 37 °C. After DNAse treatment, the TUNEL staining was performed with *In Situ Cell Death Detection Kit* (Roche, 1684795) following the manufacturer's instruction. The negative control was incubated in the

same conditions as the rest of the samples but without enzyme *Terminal deoxynucleotidyl transferase*. Subsequently, samples were treated with 50 µg RNAse /mL for 1 hour at room temperature in the dark, and stained with 6.25 µg/mL propidium iodide for 15 minutes before mounting them on slides. Samples were evaluated by means of fluorescence microscopy.

Denuded oocytes were classified as: apoptotic, cells with fragmented green nuclei; or non-apoptotic, cells with intact red nuclei.

COCs were classified as: (1) Non-apoptotic COCs, if they did not show any apoptotic cumulus cells or (2) Apoptotic COC, when apoptotic cumulus cells were observed. The percentage of apoptotic cumulus cells in the whole COC was also recorded.

In blastocysts, apoptotic cell ratio (ACR) was determined as the number of TUNEL positive blastomeres in relation to the total cell number of the blastocyst.

*2.5. Experimental design*

All the COCs and oocytes necessary for one replicate of each experiment were obtained the same day. At least three replicates of each experiment were performed.

Recovered COCs from prepubertal goat ovaries were classified in two groups depending on their morphology: Healthy (H): oocytes with homogeneous cytoplasm and compact cumulus cells, and; Early atretic (EA): oocytes with heterogeneous cytoplasm and/or cumulus cells with initial expansion (based on the classification described by Bilodeau-Goeseels and Panich, 2002). Subsequently, oocyte diameter was measured, and COCs were classified in three additional groups: A: 110-125 µm; B: 125-135 µm; and C: >135 µm (Anguita et al., 2006).

Samples used for apoptosis analysis were randomly collected from each one of the 6 experimental groups and evaluated at two points of time: before IVM (0 h) and after maturation (27 h). Apoptosis evaluation was performed both in cumulus cells and denuded oocytes by TUNEL assay (see figure 1).

COCs that were not used for apoptosis detection were fertilized in vitro after 27 h of in vitro maturation, and, at 24 hour post-insemination (hpi), presumptive zygotes were placed in droplets and cultured in vitro for additional 7 days. Each droplet contained only embryos from the same morphology and diameter group. After the in vitro culture, blastocysts obtained were fixed in paraformaldehyde 4%. Apoptosis in blastocysts was evaluated by TUNEL assay the day after, and the blastocyst cell number was recorded at the same time by propidium iodide counterstaining. The rest of the embryos were stained with Hoechst in order to determine their cell number.

*2.6. Statistical analysis*

Data was analyzed using the statistical program Graph-Pad Instat 3.01 for Windows 95 (Graph-Pad software, San Diego, California, USA). Fisher's exact test was performed to compare the proportion of TUNEL positive cells in the different groups of oocytes and cumulus cells, and to assess the developmental competence among diameter groups. The apoptotic cell ratio in blastocysts from different diameter groups was analyzed using an unpaired test with Welch's correction. Differences were considered significant when $P<0.05$.

## 3. RESULTS

### *3.1. Relationship between oocyte diameter and COC morphology and prevalence of apoptosis in immature and mature oocytes*

In table 1, apoptosis in oocytes assessed by TUNEL assay are shown. In healthy immature oocytes, apoptosis detection decreased in the largest oocytes (A: 51.6%; B: 43.3%; C: 12.1%). Unexpectedly, the apoptotic rate in 110-125 and 125-135 µm oocytes was higher in Healthy than in Early Atretic oocytes (51. 6 and 43.3% vs. 11. 7 and 19. 8%, respectively).

After maturation, a reduction of apoptosis with increasing oocyte diameter was detected in Healthy oocytes (A: 31.7%; B: 12 %; C: 0%), but not in Early Atretic oocytes. In addition, a reduction of apoptosis during maturation was observed in most groups.

### *3.2. Relationship between oocyte diameter and COC morphology and prevalence of apoptosis in immature and mature cumulus-oocyte-complexes*

Results of apoptosis in cumulus cells are shown in table 2. In Healthy immature COCs, a decrease of the percentage of apoptotic COCs with increasing oocyte diameter was detected (A: 91.5%; B: 65.7%; C: 64.9%), while no statistically significant differences between diameter groups were observed in the COCs classified as Early atretic. After IVM, an increase of the percentage of COCs showing apoptotic cumulus cells was observed in most diameter groups in both Healthy and Early atretic morphologies.

During maturation the distribution of the apoptotic cumulus cells varied: while before maturation apoptotic cumulus cells were mostly located in the outer cumulus layers, after maturation they were distributed in a more dispersed way. Moreover, after IVM apoptotic cumulus cells were distributed in a more dispersed way throughout the cumulus layers in COCs classified as

early atretic in comparison to COCs classified as Healthy (personal observation).

Table 3 shows proportion of apoptotic cumulus cells after IVM. No data before maturation is shown because compactness of cumulus layers could impair the effectiveness of the assay used. After maturation, the proportion of apoptotic cumulus cells was lower in the smallest Early Atretic COCs, which showed the higher percentage of COCs with apoptotic rate lower than 10% (A: 82.9%; B: 56.1%; C: 60.4), than in the two largest groups, where the proportion of COCs with apoptotic rate in their cumulus cells between 25 and 50% was higher (A: 8.6%; B: 38.6%; C: 37.7%). Moreover, it was observed a higher incidence of apoptotic cumulus cells in the two largest COCs from the Early Atretic classification in comparison to the Healthy ones.

*3.3. Relationship between oocyte diameter and COC morphology and prevalence of apoptosis in blastocysts.*

Embryo development, in terms of total embryos obtained and blastocyst rate, improved with increasing oocyte diameter in both morphology classes (table 4). However, Healthy oocytes yielded higher blastocyst rate in the largest group than Early Atretic oocytes from the same diameter group (14.9% vs. 5.1%, respectively) ($P<0.05$). In addition, proportion of degenerated embryos was higher in the Early Atretic class. Blastocyst quality was evaluated in terms of apoptotic rate assessed by TUNEL assay (table 4), and no differences were found among diameter groups or morphology classes.

## 4. DISCUSSION

Oocyte competence to reach blastocyst stage is related to diameter of follicle from which is recovered (bovine: Blondin and Sirard, 1995; de Wit and Kruip, 2001; Kauffold et al., 2005; Lequarre et al., 2005; Lonergan et al., 1994; buffalo: Raghu et al., 2002; goat: Crozet et al., 1995) and, at the same

time, follicle diameter determines the diameter of the oocyte that contains (bovine: Arlotto et al., 1996; goat: Crozet et al., 2000; de Smedt et al., 1994; buffalo: Raghu et al., 2002). Not only follicular diameter, but also follicular atresia has been described as an important parameter that influence oocyte developmental competence: some authors found that COCs showing mild signs of follicular atresia contained oocytes with higher developmental competence (Blondin and Sirard, 1995; de Wit et al., 2000), while others concluded that only non-atretic COCs yielded higher blastocyst rate (Corn et al., 2005; Lee et al., 2001; Yuan et al., 2005; Zeuner et al., 2003). Moreover, oocyte diameter seems to influence level of atresia in cumulus cells, as an increasing oocyte diameter is accompanied by an increasing level of atresia (de Wit and Kruip, 2001). The aim of this work was to study whether COC morphology and oocyte diameter influenced the incidence of apoptosis in oocytes and cumulus cells and their developmental competence in prepubertal goats, as well as embryo quality, in order to introduce the use of these parameters to select only the most competent oocytes.

In the present study, oocyte ability to reach blastocyst stage increased with increasing oocyte diameter, according to previous studies performed in prepubertal (Anguita et al., 2006) and adult goats (Crozet et al., 1995). In prepubertal goats, COC morphology influenced embryo development, and the developmental competence was higher in the Healthy oocytes. Our results showed that, although oocyte diameter and COC morphology separately are important parameters that determine blastocyst yield, the combination of these two parameters results in the obtaining of the highest blastocyst rate.

In this study, apoptosis in immature and mature oocytes was analyzed by TUNEL assay. To our knowledge, this is the first attempt to detect apoptosis in prepubertal goat oocytes. Oocyte diameter seemed to influence incidence of apoptosis in the Healthy oocytes, showing a higher proportion of non-apoptotic oocytes in the largest group, but not in the Early Atretic ones. Apoptosis in oocytes has been previously studied, although results can be contradictory: in cattle, Yuan et al. (2005) detected no apoptotic oocytes

before or after maturation, while Warzych et al. (2007) and Matwee et al. (2000) observed apoptosis both in mature (11.2% and 23%, respectively) and immature oocytes (1.4% and 7%, respectively); in mice, apoptosis has been observed in ovulated oocytes (Perez et al., 1999; Van Blerkom and Davis, 1998); and in human, apoptosis was observed in ovulated and in vitro-matured oocytes (Van Blerkom and Davis, 1998; Wu et al., 2000). There are some animal factors that could affect oocyte quality and incidence of apoptosis in vivo, such as age and breed of the donor, ovarian morphology, estrous cycle, body condition or diet (Gordon, 2003). In our study, oocytes analysed come from prepubertal females, while in the other studies come from adult females, which could explained differences observed in immature oocytes. However, after IVM, the apoptotic rate observed in prepubertal goat oocytes was similar to incidence of apoptosis detected in mature bovine oocytes (11.2%,Warzych et al., 2007; from 17.1 to 52.3%, Wu et al., 2000; 23%, Matwee et al., 2000). Although several studies have reported a negative effect of in vitro culture conditions in oocyte viability (Fujino et al., 1996; Takase et al., 1995), in this study oocyte apoptosis decreased during maturation in all groups analyzed, which has also been observed in prepubertal goat oocytes analyzed by Annexin V staining (data not published), in bovine oocytes (Anguita et al., 2006) and in human oocytes (Van Blerkom and Davis, 1998). Differences among studies could be due to the different media used for IVM, as suggested by (Warzych et al., 2007).

Morphology of COC has been widely used as a parameter to select the most competent oocytes (Bilodeau-Goeseels and Panich, 2002; Blondin and Sirard, 1995; Yuan et al., 2005). However, the COC morphology that gives better embryo development brings contradictory results: while some studies considered that COC with signs of early atresia are more developmental competent (Bilodeau-Goeseels and Panich, 2002; Blondin and Sirard, 1995), explained by the similarity between structural changes during oocyte degeneration and those occurring in the oocyte of the dominant follicle prior to the LH surge (Assey et al., 1994; Hyttel et al., 1997), others authors found that the COCs with no signs of atresia yielded higher blastocyst rate (Corn et

al., 2005; Yuan et al., 2005). In prepubertal goats, COCs showing signs of early atresia were less competent to reach blastocyst stage after IVM, IVF and in vitro embryo culture. Apoptosis in cumulus cells from immature COCs showed that the main difference between Healthy and Early Atretic COCs was the distribution of their apoptotic cumulus cells: while most apoptotic cumulus cells were located in the outer layers of the Healthy COCs, the distribution in Early Atretic COCs was more dispersed throughout the whole complex. In fact, this more dispersed distribution is a typical feature of mature COCs, as has been previously observed in bovine (Yuan et al., 2005), and has been related to a premature oocyte-cumulus uncoupling (de Wit et al., 2000). After maturation, the percentage of apoptotic cumulus cells was higher in Early Atretic COCs, which could explain the differences in developmental competence observed between Healthy and Early Atretic COCs.

Apoptosis incidence in blastocysts has been related to embryo quality (Hardy et al., 1989). Apoptosis plays a very important role during pre- and post-implantation development, removing abnormal cells or cells that are no longer required and controlling the embryo cell number. The protective role of apoptosis in the pre-implantation period can turn into damage if the incidence in the embryo is very high. However, it is difficult to establish a detrimental threshold of apoptosis incidence, since apoptosis has been observed in in vivo bovine, porcine, rodent (Fabian et al., 2005), primate and human blastocysts (Hardy, 1997). Therefore, consequences of apoptotic incidence in blastocyst quality are difficult to assess. In our study, apoptotic cell ratio did not vary between blastocysts from different diameter or morphology groups. Other studies using TUNEL assay in in vitro produced blastocysts showed an average apototic index similar to ours (2. 9%, Warzych et al., 2007; 3-7%, Boelhauve et al., 2005; 4-9%, Fabian et al., 2005) or a bit higher (10.3%, Pomar et al., 2005; 12%, Yuan et al., 2005; 9.8-14.2%, Vandaele et al., 2006; 12%, Anguita et al., 2006). However, none of these studies found an incidence of apoptosis higher than 15-20%, which could be considered a threshold level of detrimental incidence of apoptosis in

blastocysts. From our results, we could establish that apoptosis in oocytes and COCs did not affect blastocyst quality in terms of apoptosis incidence.

In conclusion, oocyte developmental competence in prepubertal goats is influenced by oocyte diameter and COC morphology. Apoptosis in oocytes and cumulus cells differed depending on oocyte diameter and COC morphology. Therefore, apoptosis in oocytes and cumulus cells could affect embryo development, but not embryo quality assessed by apoptosis in blastocysts. In our conditions, oocyte diameter and COC morphology are good selection parameters, and only the healthy oocytes higher than 135 µm should be use to obtained the highest blastocyst rate.

## 5. ACKNOWLEDGEMENTS

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Table 1. Apoptosis rate in immature and IVM-oocytes assessed by TUNEL assay depending on oocyte diameter and COC morphology.

| | | **Healthy** | | | **Early Atretic** | | |
|---|---|---|---|---|---|---|---|
| | | *110-125 µm* | *125-135 µm* | *>135 µm* | *110-125 µm* | *125-135 µm* | *>135 µm* |
| **Immature oocytes** | *Oocytes evaluated* | 64 | 104 | 66 | 60 | 91 | 55 |
| | *Apoptotic (%)* | 33 [a?] (51.5) | 45 [a?] (43.3) | 8 [b] (12.1) | 7 [b] (11.7) | 18 [b?] (19.8) | 14 [b?] (25.4) |
| **IVM-oocytes** | *Oocytes evaluated* | 63 | 83 | 25 | 78 | 102 | 55 |
| | *Apoptotic (%)* | 20 [a??] (31.7) | 10 [b?] (12) | 0 [b] (0) | 9 [b] (11.5) | 7 [b?] (6.9) | 5 [b?] (9.1) |

Values in the same row with different superscripts ([a, b]) differ significantly (P<0.05). Values in the same column with different superscripts ([????]) differ significantly (P<0.05).

Table 2. Distribution of apoptotic cumulus cells (CCs) assessed by TUNEL assay in immature and IVM-COCs depending on COC morphology and oocyte diameter.

| | | **Healthy** | | | **Early Atretic** | | |
|---|---|---|---|---|---|---|---|
| | | *110-125 µm* | *125-135 µm* | *>135 µm* | *110-125 µm* | *125-135 µm* | *>135 µm* |
| **Immature COCs** | *COCs evaluated* | 82 | 111 | 57 | 47 | 75 | 51 |
| | *COCs with apoptotic CCs (%)* | 75 $^{a}$ (91.5) | 73 $^{b\ ?}$ (65.7) | 37 $^{bd\ ?}$ (64.9) | 42 $^{ac}$ (89.4) | 60 $^{acd?}$ (80) | 40 $^{bcd}$ (78.4) |
| **IVM- COCs** | *COCs evaluated* | 51 | 74 | 31 | 44 | 57 | 53 |
| | *COCs with apoptotic CCs (%)* | 42 $^{ab}$ (82.3) | 60 $^{a\ ?}$ (81.1) | 28 $^{ab\ ?}$ (90.3) | 35 $^{a}$ (79.5) | 54 $^{b\ ?}$ (94.7) | 49 $^{ab}$ (92.4) |

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

Table 3. Percentage of apoptotic cumulus cells assessed by TUNEL assay in IVM- COCs depending on COC morphology and oocyte diameter.

| | | **Healthy** | | | **Early Atretic** | | |
|---|---|---|---|---|---|---|---|
| | | *110-125 µm* | *125-135 µm* | *>135 µm* | *110-125 µm* | *125-135 µm* | *>135 µm* |
| *Apoptotic COCs* | | 42 | 60 | 28 | 35 | 57 | 53 |
| *IVM-COCs with incidence of apoptotic CCs of:* | *<10 %* | 38 [a] (90.5) | 48 [a] (80) | 26 [a] (92.9) | 29 [a] (82.9) | 32 [b] (56.1) | 32 [b] (60.4) |
| | *10-25 %* | 3 [a] (7.1) | 9 [a] (15) | 2 [a] (7.1) | 3 [a] (8.6) | 22 [b] (38.6) | 20 [b] (37.7) |
| | *25-50 %* | 1 (2.4) | 1 (1.7) | 0 | 2 (3.5) | 2 (3.5) | 1 (1.9) |
| | *>50 %* | 0 | 2 (3.3) | 0 | 1 (1.7) | 1 (1.7) | 0 |

Values in the same row with different superscripts ([a, b]) differ significantly ($P<0.05$).

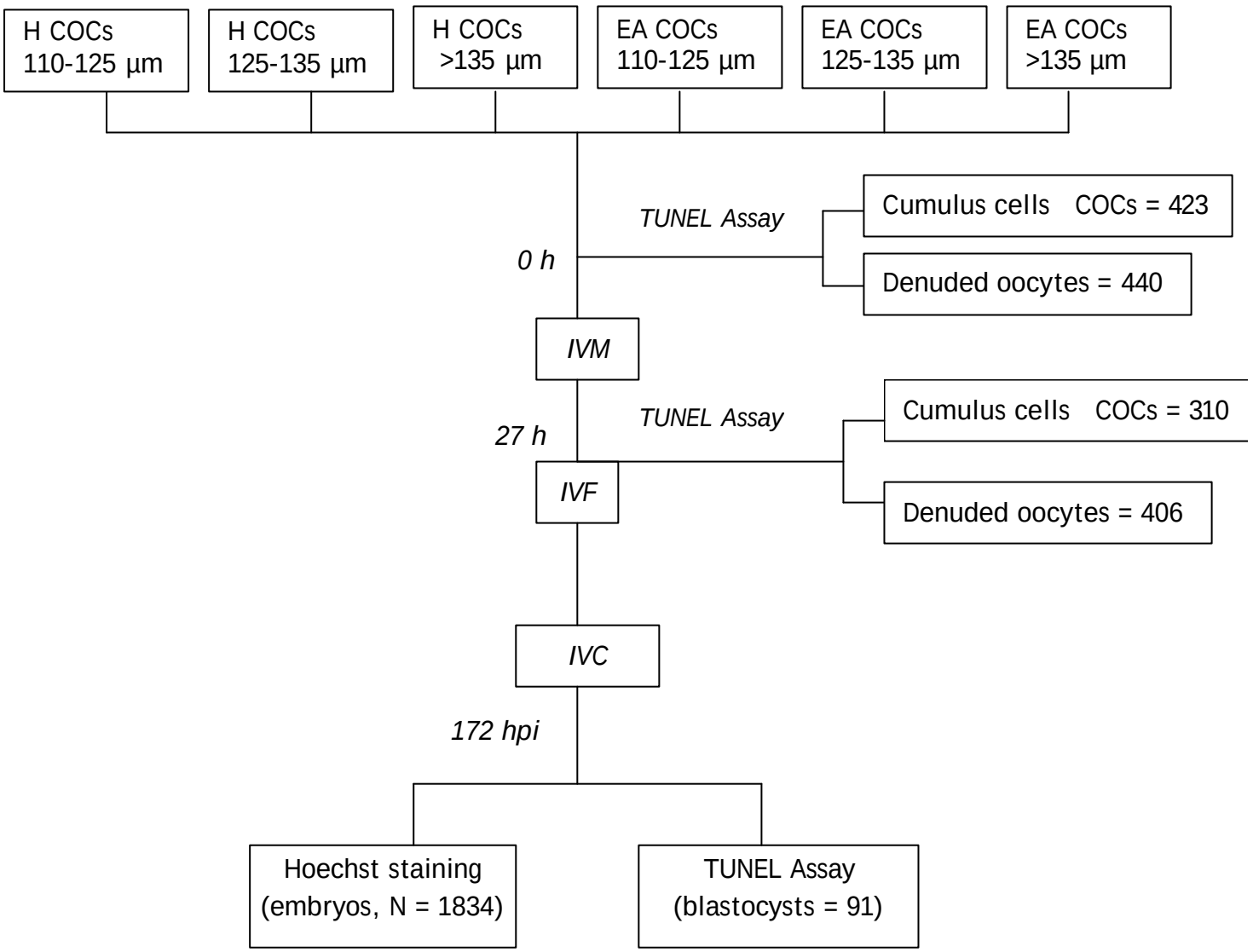
Table 4. Embryo development at 8 dpi depending on COC morphology and oocyte diameter

| | Ø | N | Total Div | 8-16 Cells | Mor | Blastos | Deg | ACR (%) |
|---|---|---|---|---|---|---|---|---|
| **Healthy** | *110-125 µm* | 222 | 71 [a] (32) | 6 [a] (2.7) | 4 [a] (1.8) | 0 [a] | 18 [ac] (8.1) | 0 |
| | *125-135 µm* | 510 | 303 [b] (59.4) | 41 [b] (8) | 20 [ab] (3.9) | 27 [b] (5.3) | 31 [c] (6.1) | 3.61 ± 4.67 |
| | *>135 µm* | 230 | 165 [c] (71.7) | 28 [bc] (12.2) | 16 [bc] (7) | 33 [c] (14.3) | 17 [ac] (7.4) | 2.52 ± 2.03 |
| **Early atretic** | *110-125 µm* | 284 | 95 [a] (33.4) | 12 [a] (4.2) | 5 [a] (1.8) | 1 [a] (0.3) | 34 [ab] (12) | 2.05 |
| | *125-135 µm* | 463 | 292 [b] (63.1) | 59 [c] (12.7) | 13 [a] (2.8) | 19 [b] (4.1) | 42 [ac] (9.1) | 3.70 ± 5.56 |
| | *>135 µm* | 215 | 145 [c] (68.8) | 38 [c] (17. 7) | 17 [c] (7.9) | 11 [b] (5.1) | 31 [b] (14.4) | 3.09 ± 2.95 |

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

Total Div: Total number of embryos; Mor: Morulae; Blastos: Blastocysts; ACR: Apoptotic cell ratio

Figure 1. Experimental design



# EFFECT OF PREVALENCE OF APOPTOSIS IN OOCYTES AND CUMULUS CELLS, ASSESSED BY ANNEXIN-V STAINING, ON EMBRYO DEVELOPMENT IN PREPUBERTAL GOATS

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**ABSTRACT**

Oocyte quality is the main factor that determine blastocyst yield. Any factor that could affect oocyte quality, such as apoptosis, could impair the subsequent embryonic development. Our aim was to investigate the incidence of apoptosis in prepubertal goat oocytes and cumulus cells, assessed by annexin-V staining, to evaluate its effect on embryo development. COCs from slaughtered females were collected and classified in groups depending on oocyte diameter: A: 110-125 µm, B: 125-135 µm and C: >135 µm. Each diameter group was classified in two groups depending on COC morphology: Healthy (H: compact cumulus layers and homogeneous cytoplasm) or Early Atretic (EA: granulated cytoplasm and/or dispersed cumulus layers). COCs were IVM for 27 h, IVF with fresh semen and IVC for 8 days after insemination. Apoptosis analyses in oocytes and cumulus cells were performed after and before maturation in all groups by Annexin V staining. Fisher's exact test showed that viable oocytes increased with diameter in the EA class both after and before maturation (Immature oocytes: A: 70.9 %; B: 75.2 %; C: 72.4 %; IVM-oocytes: A: 82.5 %; B: 95.2 %; C: 100 %), but not in the H class. Apoptotic cumulus cells in immature COCs were less predominant in the largest oocytes. There were less apoptotic cumulus cells in the H than in EA COCs. Blastocyst yield obtained from the largest oocytes was the highest. In conclusion, apoptosis in the smallest oocytes seems to be reversible. The highest blastocyst rate is obtained from the largest healthy oocytes.

Keywords: apoptosis, oocyte, goat, Annexin-V

## 1. INTRODUCTION

The study of apoptosis incidence in oocytes as indicator of oocyte quality and its effect on embryo development is of great relevance. Storage of transcripts and proteins take place in the oocyte during its growth (Crozet *et al.*, 1981) and will determine the oocyte ability to maintain embryonic development until the activation of the embryonic genome (Bachvarova, 1992; Gandolfi and Gandolfi, 2001). Therefore, storage of any molecule that could affect oocyte quality could also impair embryo development. In fact, cell survival/death regulatory molecules can be found in the stored pool of maternal mRNA in mice and human (Exley et al., 1999; Jurisicova et al., 1998; Metcalfe et al., 2004) and as a consequence they are likely to contribute in determining the fate of the embryo. Not only the oocyte itself, but any factor that could influence its quality, as cumulus cells, could affect blastocyst yield. Oocyte and cumulus cells are connected through gap junctions, which allow the interchange of molecules between these two cellular types (Tanghe *et al.*, 2002). Consequently, apoptosis incidence in cumulus cells could be reflected in a lower oocyte developmental competence. Contradictory results in this topic have been obtained: while many authors consider that early stage of atresia in cumulus cells, assessed by morphological evaluation of COCs, is reflected in a higher blastocyst rate (Blondin and Sirard, 1995; Boni et al., 2002; de Wit et al., 2000; Nicholas et al., 2005), other studies observed that apoptosis in cumulus cells, detected with TUNEL assay, affect embryo development (Corn et al., 2005; Lee et al., 2001; Yuan et al., 2005; Zeuner et al., 2003). These results suggest that COC morphology could not be related to a higher incidence of apoptosis in cumulus cells, which should be further studied.

Many approaches performed in order to find a marker of oocyte quality are based in invasive methods (Anguita et al., 2006; Bilodeau-Goeseels and Panich, 2002; De Sousa et al., 1998; Dode et al., 2006; Gutierrez-Adan et al., 2004; Santos et al., 2006; Tamassia et al., 2004; Watson et al., 2000) and as a consequence oocytes cannot be longer used for embryo production. It is

necessary to establish a relationship between the invasive methods studied and morphological characteristics that allow an evaluation of oocyte without impairing its developmental competence. In most laboratories, COC morphology is a usual parameter to select the COCs that will be used for in vitro embryo production. However, the low blastocyst rate obtained in our laboratory following this parameter selection made necessary to use additional selection parameters. In our study, we also select oocytes by diameter, since it has been previously demonstrated in many species a positive correlation between oocyte diameter and developmental competence (Anguita et al., 2006; Gandolfi and Gandolfi, 2001; Pujol et al., 2004). Moreover, oocyte diameter has been related to incidence of apoptosis in COC (de Wit and Kruip, 2001) showing that a higher oocyte diameter was accompanied by an increasing level of atresia in COC.

In normal conditions, there is an asymmetry among the outer and the inner leaflet of the cell membrane (Bretscher, 1972): phosphatidyl-coline and sphingomielin are located in the outer leaflet, and the phosphatidyl-serine and phosphatidylethanolamine are located only in the inner leaflet. However, translocation of phospatidyl-serine to the outer leaflet takes place in the early stage of apoptosis, before DNA fragmentation (revised by (van Engeland et al., 1998). Therefore, detection of phospatidyl-serine in the outer leaflet can be used as a marker of early apoptosis. Annexin V is a molecule that binds specifically to phospatidyl-serine (Andree et al., 1990; Tait et al., 1989). Annexin V cannot cross the cell membrane of viable cells, but it can enter dead cells that have lost membrane integrity and bind to the posphatydilserine present in the inner leaflet. Consequently, it is necessary to combine Annexin V staining with a vital stain, such as propidium iodide, in order to distinguish between viable and dead cells (revised by (van Engeland et al., 1998).

The objective of the present study was: 1) to evaluate the relationship of oocyte diameter and COC morphology with oocyte and cumulus cells apoptosis; 2) to establish whether COC morphology was related to a

differential incidence of apoptosis in oocytes or cumulus cells; 3) to study the relationship between oocyte and cumulus cells apoptosis with embryo development. In all experiments, apoptosis was assessed by Annexin-V staining.

## 2. MATERIAL AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

### *2.1. Oocyte collection and IVM*

Ovaries from prepubertal goats, approximately 2 months old, were recovered from a local slaughterhouse and transported within 2 hours to the laboratory at 38.5 ºC in PBS (Dulbecco's phosphate-buffered saline, P-4417) with 50 µg/mL gentamycin (G-3632). Ovaries were washed three times and COCs (cumulus-oocyte complexes) were recovered by slicing in TCM199 (M-2520) supplemented with 2.2 mg/mL $NaHCO_3$, 2% (v/v) steer serum (Donor Bovine Serum? , CanSera, Canada) and 50 ?g/mL gentamycin.

COCs were matured in TCM199 medium (M-7528) supplemented with 275 ?g/mL sodium pyruvate (P-3662), 146 µg/mL L-glutamine (G-5763), 10% (v/v) steer serum, 10 µg/mL o-LH (L-5269), 10 µg/mL o-FSH (F-4520), 1 µg/mL 17-ß estradiol (E-2257), 400 µM cysteamine (M-9768) and 50 µg/mL gentamycin (G-3632). Groups of 20-25 COCs were transferred to 100 µl microdrops of maturation medium under mineral oil (M-3516) and incubated for 27 hours at 38.5 ºC in a humidified air atmosphere with 5% $CO_2$.

### *2.2. Sperm capacitation and IVF*

Fresh semen was collected from two males of proven fertility and transported to the laboratory at 38.5 ºC within 30 minutes. Motility of the ejaculates was evaluated under a stereomicroscope. Motile sperm fraction was separated by the swim-up method (Parrish *et al.*, 1986), whereby 70 µL of

sperm were layered below 2 mL of mDM (Defined medium, (Brackett and Oliphant, 1975); modified by (Younis et al., 1991), and incubated for 45 min at 38.5 ºC in a humidified air atmosphere with 5% $CO_2$. After incubation, the supernatant was recovered and centrifuged at 170 x g for 3 minutes. The pellet was resuspended in mDM (1:1; v/v) containing heparin (H-3149) and ionomycin (I-0634) (Final concentration: 10 µg/mL heparin and 200 nM of ionomycin) and incubated for 15 min in a humidified air atmosphere with 5%$CO_2$ and 38.5 ºC (Wang *et al.*, 2002). Sperm concentration was determined using a Neubauer chamber cell.

After maturation, groups of 20 to 25 oocytes were transferred to 100 µL microdrops of modified Tyrode's medium (TALP) as described by Parrish et al. (1986), supplemented with 1 µg/mL hypotaurine (H-1384) and 0.3 mg/mL glutathione (G-6013) under mineral oil. Oocytes were co-cultured with capacitated spermatozoa in a final concentration in the microdrop of 4 x $10^6$ sperm cells/mL.

*2.3. In vitro embryo culture*

At 24 hours post-insemination, presumptive zygotes were washed to remove both remnant cumulus cells and attached sperm cells. Groups of 18-25 embryos were placed in 50 µl droplets of SOF medium (Synthetic Oviductal Fluid, (Holm et al., 1999) and covered with mineral oil. The embryos were incubated at 38.5°C in 5% $CO_2$, 5% $O_2$ and 90 % $N_2$ for 7 days. At 48 hpi 0.1 µL fetal bovine serum (F-7524) per embryo was added to the medium. At the end of the culture period (8 days post-insemination), total cell number of embryos was evaluated by Hoechst 33342 staining (B-2261) under a fluorescence microscope.

*2.4. Annexin-V staining*

Detection of apoptosis was performed with Annexin-V-FLUOS Staining kit (Roche, 1858777). During the whole process the reagents were kept at 38.5°C.

Staining was performed following the manufacturer's instructions with minor modifications. Briefly, samples were placed in 35 µl droplets containing Annexin V buffer, Annexin V/FITC, PI and 1 mg/ml Hoechst 33342, and incubated for 15 minutes at 38°C in the dark. Cells were counterstained with propidium iodide (PI), a membrane impermeable stain, to distinguish between live cells and dead cells. After incubation cells were mounted on siliconized slides with vaseline bridges and observed under a fluorescence microscope.

Denuded oocytes were classified in three groups, representing (1) non-apoptotic oocytes, with intact Hoechst positive nuclei and no annexin V staining; (2) early apoptotic oocytes, with intact Hoechst positive nuclei and a homogeneous annexin positive signal in the membrane; and (3) necrotic oocytes which showed PI positive red nuclei, which is indicative for membrane damage. In this last group, there were occasionally signs of annexin V staining in the membrane. Oocytes showing a discontinuous green signal that came from the remaining membrane of cumulus cells projections (Van Blerkom and Davis, 1998) were also considered to be non-apoptotic oocytes.

COCs were evaluated as a whole mount preparation, and we evaluated the proportion of the stained cumulus cells in the COC. The classification was the following: (1) Healthy COCs, with no cells stained; (2) Apoptotic COCs, with cells stained in with annexin V and, ocassionally, with propidium iodide. It was also recorded if the percentage of stained cumulus cells represented less than 25%, from 25 to 50%, from 50 to 75% or more than 75% of the total cumulus cells.

*2.5. Experimental design*

All the COCs and oocytes necessary for one replicate of each experiment were obtained the same day. At least three replicates of each experiment were performed.

Recovered COCs from prepubertal goat ovaries were classified in two groups depending on their morphology: Healthy (H): oocytes with homogeneous cytoplasm and compact cumulus cells, and; Early atretic (EA): oocytes with heterogeneous cytoplasm and/or cumulus cells with initial expansion. Subsequently, oocyte diameter of COCs corresponding on each morphological category was measured, and COCs were classified in three additional groups: group A: 110-125 µm; group B: 125-135 µm; and group C: >135 µm (Anguita et al., 2006).

Samples used for apoptosis analysis were randomly collected from each one of the 6 experimental groups and evaluated at two points of time: before IVM (0 h) and after maturation (27 h). Apoptosis evaluation was performed both in cumulus cells and denuded oocytes by Annexin-V staining (see figure 1). COCs that were not used for apoptosis detection were fertilized in vitro after 27 h of in vitro maturation, and at 24 hour post-insemination (hpi) presumptive zygotes were cultured in vitro for additional 7 days. Each droplet contained only embryos from the same morphology and diameter group. After the in vitro culture, total number cell of embryos was evaluated by Hoechst staining.

*2.6. Statistical analysis*

Data was analyzed using the statistical program Graph-Pad Instat 3.01 for Windows 95 (Graph-Pad software, San Diego, California, USA). Fisher's exact test was performed to compare the proportion of Annexin-V positive cells in the different groups of oocytes and COC, and to assess the developmental competence among diameter groups. Data of cell mean per

blastocyst were evaluated using analysis of variance with Tukey's post-test. Differences were considered significant when $P<0.05$.

## 3. RESULTS

### *3.1. Relationship between oocyte diameter and COC morphology and prevalence of apoptosis in immature and mature oocytes*

Annexin-V staining in immature oocytes (table 1) showed that the percentage of apoptotic oocytes decreased with oocyte size in the EA class (A: 42.6%; B: 30.3%; 21%), but not in the H class (A: 29.1%; B: 24.8%; 27.5%). No differences in the percentage of apoptotic oocytes were detected in oocytes of the same diameter but different morphology group.

After maturation (table 1) a clear decrease of apoptotic oocytes (A: 17.5%; B: 4.8%; C: 0%) was observed with increasing oocyte diameter in the EA class. However, in the H class, the percentage of apoptotic oocytes was lower in the 125-135 µm oocytes (A: 5.5%; B: 0%; C: 3.7%). A reduction of the incidence of apoptosis was also observed during maturation whatever the diameter or the morphology group.

We observed that most apoptotic oocytes were at the latest stages, and that necrotic ocytes were only detected in the EA class (data not shown).

### *3.2. Relationship between oocyte diameter and COC morphology and prevalence of apoptosis in immature and mature cumulus-oocyte-complexes*

Results are shown in table 2. At collection time, in both morphology groups, the percentage of apoptotic cumulus cells decreased in the largest COCs, and the percentage of COCs with less incidence of apoptosis was higher in class H when compared to the same diameter group from class EA (80.5, 82.5 and 94.6% vs. 40.7, 48 and 60%, respectively). After maturation, the

incidence of apoptosis was lower in the largest Healthy oocytes. Moreover, the percentage of COCs with more than 50% of apoptotic cumulus cells was higher in >135 µm COCs from class EA when compared to the same diameter group from class H.

The percentage of apoptotic cumulus cells varied during maturation only in Healthy oocytes, and we detected a decrease in the percentage of COCs with less than 25% of apoptotic cumulus cells, and an increase in the percentage of COCs with 25-50% of apototic cumulus cells after maturation.

We observed that most of apoptotic cumulus cells in immature COCs classified as Healthy was at the earliest stages of apoptosis, while in COCs classified as Early atretic, most of apoptotic cumulus cells was at late stage of apoptosis or necrosis. In addition, after IVM, apoptotic cumulus cells detected in the largest oocytes were at an earliest stage of apoptosis in both morphology classes, when compared to the other diameter groups. We detected no necrotic COC in the largest group from the H class; however, the proportion of necrotic COCs in the same diameter group from the EA class was 30% (data not shown).

*3.3. Relationship between oocyte diameter and COC morphology and prevalence of apoptosis in blastocysts.*

Results of embryo development are shown in table 3. We observed a clear relationship between oocyte diameter and embryo development in terms of total embryos obtained and blastocyst rate in both morphology classes, although in class EA no differences among 125-135 µm and >135 µm oocytes were observed. In class H the blastocyst rate obtained in the largest group was statistically higher than the blastocyst rate obtained by the same diameter group in class EA (14.9% vs. 5.1%, respectively).

Blastocyst quality was evaluated in terms of percentage of hatched blastocysts, and number of cells per blastocyst (table 3). No differences in percentage of hatched blastocysts were found among diameter groups or morphology classes. Cell number per blastocysts did not differ among diameter nor do morphology groups, but the number of cells in the blastocysts derived from the largest oocytes tended to be higher in the healthy group ($P<0.10$)

## 4. DISCUSSION

In this study we have investigated the apoptosis incidence in oocytes and cumulus cells related to morphological characteristics, in order to know whether apoptosis incidence could affect blastocyst yield and quality. For that purpose we have used Annexin-V staining, which detects translocation of phosphatidylserine, a membrane phospholipid that flips from the inner to the outer leaflet of the plasma membrane during early stage of apoptosis (Martin et al., 1995; van Engeland et al., 1998). In addition, Annexin-V staining, in combination to propidium iodide staining, lets us to distinguish between viable and dead cells.

Apoptosis in prepubertal goat oocytes assessed by Annexin-V was detected in both immature and IVM-oocytes. Our results showed a reduction of incidence of apoptosis during maturation, which is contradictory to several studies in mouse, that reported a negative effect of culture in vitro in terms of oocyte apoptosis detected by TUNEL assay (Fujino et al., 1996; Takase et al., 1995). However, (Van Blerkom and Davis, 1998) detected an increase and a decrease of apoptosis, assessed by Annexin-V staining, during culture in vitro in murine and human oocytes, respectively, while (Anguita et al., 2006) reported a decrease of bovine oocyte apoptosis during in vitro maturation. Discrepancies among authors may be caused by a differential incidence of apoptosis between species or differences in the media composition, as was suggested by Warzych et al. (2007). However, other hypotheses that could

explain reduction of apoptosis during in vitro maturation could be related to the removal of oocyte from a follicular environment that may be promoting apoptosis, the manipulation-induced apoptosis started in immature oocytes and reverted during in vitro culture in specific conditions, or even a higher sensitivity of immature oocytes to suffer apoptosis in comparison to mature oocytes. In fact, in studies of oocyte cryopreservation, a reduced resistance to cryopreservation in GV-oocytes in comparison to MII-oocytes has been observed (Otoi et al., 1995; Parks and Ruffing, 1992), which may be related to the higher incidence of apoptosis in immature oocytes, although further studies should be performed in this matter.

Apoptosis is a regulatory mechanism that allows the maintenance of homeostasis and the elimination of cells that are no longer required, and has been also suggested as the mechanism to remove oocytes that have resumed meiosis inside the follicle. However, in this study, oocyte nuclear stage did not influence prevalence of apoptosis in oocytes after or before maturation, which would indicate that apoptosis detected in prepubertal goat oocytes is not a consequence of meiotic resumption inside the follicle. Only Early Atretic oocytes larger than 135 µm showed a higher incidence of apoptosis in oocytes at GV stage. These results are not in accordance with previous studies, which detected higher apoptotic rate in "immature" oocytes that had resumed meiosis (Anguita et al., 2006; Warzych et al., 2007). However, these studies evaluated apoptotic rate in morphologically healthy oocytes, while we only observe and effect of nuclear stage in morphologically Early Atretic oocytes, which could explain the differences among studies.

Incidence of apoptosis in cumulus cells can be a good indicator of oocyte developmental competence (Corn et al., 2005; Lee et al., 2001; Yuan et al., 2005), due to the bidirectional communication established between cumulus cells and oocytes through gap junctions (de Loos *et al.*, 1991). Cumulus cells play an important role in the regulation of nuclear and cytoplasmic maturation in the oocyte (Tanghe *et al.*, 2002) and in protecting oocytes against the oxidative stress-induced apoptosis (Tatemoto *et al.*,

2000). In addition, presence of cumulus cells during in vitro maturation is essential to improve blastocyst rate (Hashimoto *et al.*, 1998). Several studies have related mild signs of atresia in COCs with higher oocyte developmental competence (Blondin and Sirard, 1995; Boni et al., 2002; de Wit et al., 2000; Nicholas et al., 2005), while other studies reported lower blastocyst rate from early atretic COCs (Corn et al., 2005; Lee et al., 2001; Yuan et al., 2005; Zeuner et al., 2003). The use of different parameters to evaluate apoptosis in a cell could explain the contradictory results from those studies. For that reason, we investigated the relationship between morphological characteristics and apoptosis incidence in COCs from prepubertal goats. We observed that immature COCs classified morphologically as Early Atretic really showed a higher percentage of apoptotic cells than those classified as Healthy. Moreover, Early Atretic COCs yielded lower blastocyst rate than the Healthy ones (3.2% vs. 6.2%, respectively). Our results enhance the importance of the use of COC morphology as a selection parameter in prepubertal goats. In addition to morphological selection, classification of COCs by oocyte diameter was also useful as an additional selection parameter, since the largest COCs presented the lowest incidence of apoptosis, and their apoptotic cumulus cells were at the earliest stage of apoptosis.

Our results showed a higher blastocyst rate achieved from the largest oocytes, which is in agreement to previous studies which observed a relationship between oocyte diameter and developmental competence (Anguita et al., 2006; Gandolfi and Gandolfi, 2001). In prepubertal goats, the use of early atretic COCs impaired embryo development from oocytes highest than 135 µm. Taken together our results of embryonic development and apoptosis in oocytes and cumulus cells, it seems that apoptosis in cumulus cells is an important factor in determining differences in blastocyst yield observed among oocytes of different diameter and oocytes of different morphology. Thus, a high embryonic development is related to a reduced incidence of apoptosis in both mature and immature COCs and a higher prevalence of early stage of atresia in the apoptotic cumulus cells. These

results suggest that apoptosis in cumulus cells could influence oocyte quality and, therefore, its developmental competence. However, oocyte ability to develop into an embryo depends also on its capability to complete nuclear and, more important, cytoplasmic maturation. It is widely accepted that incompetent oocytes do not reach the blastocyst stage because of a lack of accumulation of RNA and proteins, necessary to maintain embryonic development beyond the embryonic genome activation (Bachvarova, 1992). Moreover, a recent study performed in our laboratory showed that $p34^{cdc2}$ expression and MPF activity (an important regulator of oocyte maturation) influenced oocyte capability to reach blastocyst stage (Anguita et al., 2006). It is possible that MPF could also regulate some biochemical ways of apoptosis through the regulation of MAPK activity, as has been observed in starfish (Chiba, 2004; Greenwood and Gautier, 2005) and *Drosophila* (Greenwood and Gautier, 2005). Therefore, it is more likely that the combination of several factors, rather that only one, determines oocyte developmental competence.

No information about the effect of oocyte diameter or morphology of COC in embryo quality has been found in prepubertal goats. In this study, we have tried to evaluate embryo quality in terms of hatching rate and cell mean number per blastocyst. Our results showed no differences in the total hatched blastocysts among diameter and morphology groups, and we obtained a mean hatching rate at 8 dpi of 52.7%. Studies performed in different species showed variable hatching rates: in cattle, Nedambale et al. (2006) reported a mean hatching rate of 69%, while Balasubramanian and Rho (2006) obtained only 16.8% of hatched blastocysts; in sheep, a mean of 60% of blastocysts hatched at 7 dpi (Garcia-Garcia *et al.*, 2006). Taking into account that we used oocytes from prepubertal animals to produce blastocysts, our hatching rate indicates that the quality of the blastocysts obtained was not substantially lower than those obtained from adult animals. Although no differences in the mean cell number were found between blastocyst of different groups, we observed a tendency to increase the mean cell number in the blastocysts coming from the largest oocytes classified as Healthy. We obtained a mean cell number of 110, which is similar to the mean cell number reported by

Koeman et al. (2003) (120 ± 32) in blastocysts obtained from prepubertal goat oocytes. The fact that Koeman et al. (2003) did not detect differences between blastocysts obtained from prepubertal and adult goats reflects that blastocyst quality in goats does not depend on animal age, and validates the use of prepubertal goats as oocyte donors for embryo production.

In conclusion, in this study and in our experimental conditions, oocyte quality, assessed by incidence of apoptosis in prepubertal goat oocytes and cumulus cells, influenced blastocyst yield but not blastocyst quality. We confirmed that COC morphology in prepubertal goats was related to a differential incidence of apoptosis in cumulus cells and oocyte developmental competence. We observed an effect of oocyte diameter on prevalence of apoptosis in oocytes and cumulus cells, as well as in oocyte developmental competence. Therefore, selection of prepubertal goat oocytes according to oocyte diameter and COC morphology is a useful tool to select the COCs with a lower incidence of apoptosis and a higher competence to reach blastocyst stage.

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Table 1. Apoptosis rate in immature and IVM- oocytes assessed by Annexin-V staining depending on oocyte diameter and COC morphology.

| | | Healthy | | | Early Atretic | | |
|---|---|---|---|---|---|---|---|
| | | *110-125 µm* | *125-135 µm* | *>135 µm* | *110-125 µm* | *125-135 µm* | *>135 µm* |
| **Immature oocytes** | *Oocytes evaluated* | 79 | 125 | 69 | 68 | 109 | 62 |
| | *Apoptotic oocytes (%)* | 23 $^{ab}$ (29.1) | 31 $^{b}$ (24.8) | 19 $^{ab}$ (27.5) | 29 $^{a}$ (42.6) | 33 $^{ab}$ (30.3) | 13 $^{b}$ (21) |
| **IVM-oocytes** | *N* | 73 | 86 | 27 | 63 | 104 | 47 |
| | *Apoptotic oocytes (%)* | 4 $^{a}$ (5.5) | 0 $^{c}$ | 1 $^{abc}$ (3.7) | 11 $^{b}$ (17.5) | 5 $^{ac}$ (4.8) | 0 $^{ac}$ |

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

Table 2. Apoptotic rate in cumulus cells assessed by Annexin-V staining and their distribution in immature and IVM- COCs depending on COC morphology and oocyte diameter

| | | **Healthy** | | | **Early Atretic** | | |
|---|---|---|---|---|---|---|---|
| | | *110-125 µm* | *125-135 µm* | *>135 µm* | *110-125 µm* | *125-135 µm* | *>135 µm* |
| Immature COCs | *COCs evaluated* | 87 | 103 | 56 | 54 | 77 | 70 |
| | *Apoptotic COCs (%)* | 86 $^{ab}$ (98.8) | 97 $^{a}$ (94.2) | 55 $^{a}$ (98.2) | 54 $^{ab}$ (100) | 77 $^{b}$ (100) | 70 $^{ab}$ (100) |
| | *25 %* | 70 $^{a??}$ (81.4) | 85 $^{a??}$ (87.6) | 53 $^{b??}$ (96.4) | 22 $^{c}$ (40.7) | 37 $^{c}$ (48.05) | 42 $^{d}$ (60) |
| | *25-50 %* | 4 $^{a?}$ (4.6) | 6 $^{a?}$ (6.2) | 1 $^{a??}$ (1.8) | 16 $^{b}$ (29.6) | 16 $^{b}$ (20.8) | 12 $^{b}$ (17.1) |
| | *50-75 %* | 7 $^{ab}$ (8.1) | 3 $^{b}$ (3.1) | 0 $^{c}$ | 9 $^{a}$ (16.7) | 9 $^{a}$ (11.7) | 6 $^{ab}$ (8.6) |
| | *>75 %* | 5 $^{ab}$ (5.8) | 3 $^{b}$ (3.1) | 1 $^{b}$ (1.8) | 7 $^{ac}$ (13) | 15 $^{c}$ (19.5) | 10 $^{c}$ (14.3) |
| IVM- COCs | *COCs evaluated* | 59 | 75 | 50 | 45 | 65 | 60 |
| | *Apoptotic COCs (%)* | 59 (100) | 75 (100) | 50 (100) | 45 (100) | 65 (100) | 60 (100) |
| | *25 %* | 29 $^{a?}$ (49.1) | 45 $^{ab?}$ (60) | 37 $^{b?}$ (74) | 21 $^{a}$ (46.7) | 37 $^{ab}$ (57.9) | 32 $^{ac}$ (53.3) |
| | *25-50 %* | 17 $^{?}$ (28.8) | 17 $^{?}$ (22.7) | 13 $^{??}$ (26) | 9 (20) | 10 (15.4) | 9 (15) |
| | *50-75 %* | 7 $^{a}$ (11.9) | 8 $^{a}$ (10.7) | 0 $^{b}$ | 9 $^{a}$ (20) | 9 $^{a}$ (13.8) | 9 $^{a}$ (15) |
| | *>75 %* | 6 $^{a}$ (10.2) | 5 $^{ab}$ (6.7) | 0 $^{b}$ | 6 $^{a}$ (13.3) | 9 $^{a}$ (13.8) | 10 $^{a}$ (16.7) |

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

Table 3. Embryo development at 8 dpi depending on COC morphology and oocyte diameter

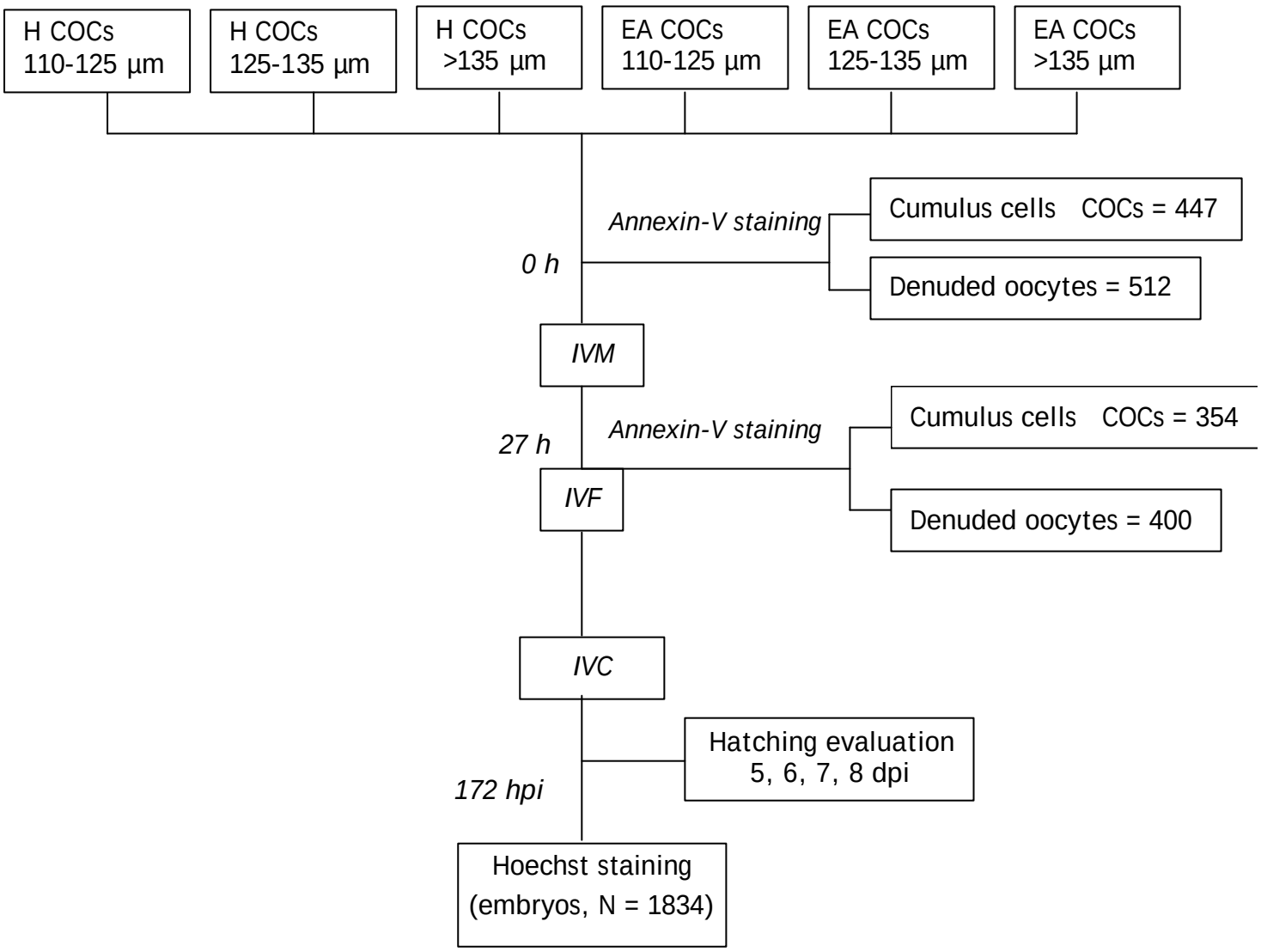
| | Ø (µm) | N | Total Div (%) | 8-16 Cells (%) | Mor (%) | Blast. (%) | Deg (%) | Hatched Blast.* | Cell Mean Blast. |
|---|---|---|---|---|---|---|---|---|---|
| **Healthy** | *110-125 µm* | 222 | 71 [a] (32) | 6 [a] (2.7) | 4 [a] (1.8) | 0 [a] | 18 [ac] (8.1) | 0 | |
| | *125-135 µm* | 510 | 303 [b] (59.4) | 41 [b] (8) | 20 [ab] (3.9) | 27 [b] (5.3) | 31 [c] (6.1) | 14/27 (51.85) | 92.52 ± 51.87 |
| | *>135 µm* | 230 | 165 [c] (71.7) | 28 [bc] (12.2) | 16 [bc] (7) | 33 [c] (14.3) | 17 [ac] (7.4) | 16/33 (48.48) | 128.90 ± 81.40 |
| **Early atretic** | *110-125 µm* | 284 | 95 [a] (33.4) | 12 [a] (4.2) | 5 [a] (1.8) | 1 [a] (0.3) | 34 [ab] (12) | 1/1 (100) | 146 |
| | *125-135 µm* | 463 | 292 [b] (63.1) | 59 [c] (12.7) | 13 [a] (2.8) | 19 [b] (4.1) | 42 [ac] (9.1) | 9/19 (47.37) | 106.7 ± 77.12 |
| | *>135 µm* | 215 | 145 [c] (68.8) | 38 [c] (17. 7) | 17 [c] (7.9) | 11 [b] (5.1) | 31 [b] (14.4) | 8/11 (72.72) | 107.38 ± 67.73 |

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

* These results are expressed as the hatched blastocysts related to the total number of blastocysts

N: oocytes inseminated; Mor: Morulae; Blast: Blastocysts; Deg: Degenerated embryos.

Figure 1. Experimental design



# 8. DISCUSIÓN GENERAL

La producción *in vitro* de blastocistos a partir de ovocitos de cabras prepúberes (6%; Koeman et al., 2003) es bastante ineficiente, en comparación con los resultados obtenidos en cabras adultas (33%, Keskintepe et al., 1996), o en otras especies como el bovino (40%, revisado por Lonergan et al., 2003). Se ha comprobado en numerosas especies que el uso de hembras prepúberes como donantes de ovocitos perjudica el desarrollo embrionario posterior (revisado por Armstrong, 2001), debido principalmente a deficiencias en su maduración citoplasmática (Damiani et al., 1996; Salamone et al., 2001). Sin embargo, se ha logrado obtener blastocistos, gestaciones y nacimientos a partir de ovocitos de hembras prepúberes (Armstrong et al., 1992). En numerosas especies, incluido el caprino, se ha observado que la competencia del ovocito se incrementa con el tamaño folicular (vaca: Lonergan et al., 1994; Blondin y Sirard, 1995; Lequarre et al., 2005; Kauffold et al., 2005; cabra: Crozet et al., 1995; búfalo: Raghu et al., 2002; oveja: Ledda et al., 1999), y éste, a su vez, se correlaciona con el diámetro ovocitario (vaca: Arlotto et al., 1996; cabra: De Smedt et al., 1994; Crozet et al., 2000; búfalo: Raghu et al., 2002; yegua: Goudet et al., 1997). Por lo tanto, el crecimiento del ovocito es indispensable para que éste adquiera competencia para mantener el desarrollo embrionario. En cabras adultas, los folículos mayores a 5 mm de diámetro son los que contienen los ovocitos que dan lugar a un mayor porcentaje de blastocistos (Crozet et al., 1995). Sin embargo, la mayoría de folículos presentes en los ovarios de cabras prepúberes no superan los 3 mm de diámetro (Martino et al., 1994), lo que podría indicar que el menor porcentaje de desarrollo embrionario obtenido a partir de ovocitos de cabras prepúberes podría ser consecuencia de la mayor proporción de ovocitos que todavía no han completado su crecimiento, pero el potencial para el desarrollo de los ovocitos de cabras prepúberes podría ser el mismo que el de los ovocitos de cabras adultas en el mismo estadío de crecimiento. Por otro lado, en diversas especies se ha observado que el diámetro de los ovocitos obtenidos a partir de folículos del mismo diámetro es menor en hembras

prepúberes que en adultas (bovino: Gandolfi et al., 1998; ovino: Ledda et al., 1999). El objetivo de esta tesis fue estudiar la competencia de los ovocitos de cabras prepúberes a nivel meiótico, de fecundación y de desarrollo embrionario, dependiendo de su diámetro y, así, poder determinar la utilidad del diámetro ovocitario como un parámetro de selección de la calidad de los ovocitos. Por otro lado, el diámetro ovocitario también ha sido utilizado para investigar qué papel podrían desempeñar el MPF y la apoptosis en la adquisición de la competencia ovocitaria, en un intento por entender un poco más qué factores pueden estar influyendo en la maduración citoplasmática del ovocito.

Se sabe que el MPF juega un papel muy importante en la progresión de la maduración meiótica del ovocito, ya que es el responsable de la desestructuración de la lámina nuclear (Peter et al., 1990a) y de los nucleolos (Peter et al., 1990b), de la condensación cromosómica (Moreno y Nurse, 1990) y de la reorganización de los microfilamentos (Morgan et al., 1989) y de la red de filamentos intermedios (Chou et al., 1990). Nuestros resultados muestran que, en cabras prepúberes, la competencia meiótica de los ovocitos, así como la competencia para el desarrollo embrionario, se adquiere a medida que aumenta la acumulación de Ciclina B1, $p34^{cdc2}$ y la actividad del MPF en el ovocito. Nuestros resultados concuerdan con otros estudios en los que se observó que los dos componentes del MPF, la $p34^{cdc2}$ y la Ciclina B1, se van acumulando durante el crecimiento del ovocito y alcanzan niveles máximos a medida que el ovocito adquiere competencia meiótica (ratón: Mitra y Schultz, 1996; de Vántery et al., 1996; cabras: Dedieu et al., 1998). Sin embargo, la relación observada en cabras prepúberes entre la concentración y actividad del MPF y la competencia para el desarrollo de los ovocitos indica que, además del importante papel que juega en la progresión meiótica, el MPF también jugaría un papel importante en la regulación de la maduración citoplasmática del ovocito. Borsuk et al. (1991) y Naito et al. (1992) fueron los primeros en sugerir la participación del MPF en la maduración citoplasmática al observar que los niveles de MPF tras la MIV estaban relacionados con la capacidad del ovocito para descondensar la cabeza del espermatozoide y

formar el pronúcleo masculino. Por otro lado, la menor competencia de los ovocitos de hembras prepúberes también ha sido relacionada con una menor actividad del MPF en comparación con ovocitos de hembras adultas (bovino: Salamone et al., 2001; ovino: Ledda et al., 2001).

En cabras prepúberes, la acumulación de mRNA de $p34^{cdc2}$ y Ciclina B1 no parece ser la responsable de la competencia ovocitaria, ya que los niveles fueron similares en todos los diámetros de ovocitos estudiados. En cambio, sí se observaron diferencias en cuanto a los niveles de RNA de $p34^{cdc2}$ y Ciclina B1 almacenado en el ovocito antes de la maduración, pero no después, mientras que las diferencias en la cantidad de proteína $p34^{cdc2}$ se detectaron después de la maduración, pero no antes. Estos resultados parecen indicar que la acumulación de transcritos de las dos subunidades del MPF para su utilización posterior determinará la actividad del MPF y, por lo tanto, la adquisición de competencia meiótica y, posiblemente, de competencia para el desarrollo. Sin embargo, la competencia del ovocito no sólo está determinada por su capacidad para almacenar todos los factores necesarios para el mantenimiento de la actividad celular hasta la activación del genoma embrionario, sino también en su capacidad de movilizar los transcritos almacenados y traducirlos a proteínas, como se demuestra en la menor cantidad de RNA total detectado en los ovocitos más competentes después de la maduración.

En general, nuestros resultados muestran que a lo largo de la maduración *in vitro* se produjo acumulación de RNA de las subunidades del MPF, sobretodo en los ovocitos más incompetentes; sin embargo, esta acumulación no condujo a una mayor progresión meiótica en estos ovocitos. De Vantéry et al. (1997) observaron que la inyección de mRNA $p34^{cdc2}$ en ovocitos meióticamente incompetentes daba lugar a la acumulación de proteína $p34^{cdc2}$ en niveles similares a los ovocitos competentes, pero no se reiniciaba la meiosis. Estos resultados sugieren que otros factores que probablemente intervienen en la regulación del MPF pueden estar en deficiencia en los ovocitos que todavía no han completado su crecimiento.

Diversas hipótesis pueden explicar los resultados: 1) aunque se produzca acumulación de $p34^{cdc2}$, es posible que el ovocito incompetente no sea capaz de producir las modificaciones post-traduccionales que permitirán la asociación de la $p34^{cdc2}$ y la Ciclina B1 para formar el pre-MPF (Kanatsu-Shinohara et al., 2000), posiblemente debido a la incapacidad para que el $p34^{cdc2}$ sea fosforilado, paso indispensable para que la subunidad catalítica pueda asociarse con el pool endógeno de Ciclina B1 (de Vantéry et al., 1997); 2) la acumulación de $p34^{cdc2}$ es suficiente para formar el heterodímero con Ciclina B1, pero su activación puede estar comprometida si el ovocito no ha sido capaz de acumular factores necesarios para su regulación, como la fosfatasa Cdc25 o las kinasas Wee (Goudet et al., 1998; revisado por Jones, 2004); 3) es posible que la translocación del MPF del citoplasma al núcleo, donde se encuentran algunos de sus sustratos, como la histona H1, esté comprometida en ovocitos incompetentes (Kanatsu-Shinohara et al., 2000). Está translocación está regulada por la fosforilación de la Ciclina B1, que impide que el *nuclear export sequence* sea reconocido por el *nuclear export factor CRM1* (Yang et al., 1998), produciendo así su acumulación en el núcleo; y 4) la incompetencia meiótica de los ovocitos puede estar ligado a la inhabilidad para activar la MAPK, que es necesaria para dar lugar al reinicio de la meiosis en muchas especies. No obstante, en cabras la activación de la MAPK se produce después de la activación del MPF y la ruptura de vesícula germinal, al igual que en cerdos (Inoue et al., 1995) y ratón (Verlhac et al., 1994), con lo que esta hipótesis queda descartada. En cambio, se ha comprobado que los ovocitos caprinos meióticamente incompetentes contienen menos cantidad de Cdc25 fosfatasa que los ovocitos competentes (Gall et al., 2002); sin embargo, es necesario realizar más estudios para comprobar si algunos de los factores de regulación del MPF mencionados en las otras hipótesis intervienen también en la adquisición de competencia meiótica y para el desarrollo.

En cabras prepúberes la capacidad del ovocito para progresar meióticamente hasta el estadío de metafase II, ser fecundado normalmente, dividirse y alcanzar el estadío embrionario de blastocisto aumenta con el

diámetro ovocitario, hecho que ha sido previamente observado en numerosas especies (vaca: Arlotto et al., 1996; cabra: De Smedt et al., 1994; Martino et al., 1994; Crozet et al., 2000; búfalo: Raghu et al., 2002; yegua: Goudet et al., 1997). Sin embargo, nuestros resultados también muestran que el estadío meiótico de los ovocitos inmediatamente después de su liberación de los folículos varía con el diámetro; así, los ovocitos de mayor tamaño presentaban una gran proporción que ya habían reanudado la meiosis dentro del folículo. Los ovocitos que se encuentran dentro de los folículos están en parada meiótica debido a sustancias inhibidoras de la meiosis que se encuentran en el líquido folicular y que llegan al ovocito a través de las células del cumulus (Dekel, 1988). Únicamente se produce la reanudación de la meiosis en el ovocito que contiene el folículo dominante después del pico de LH, o en folículos atrésicos debido a la expansión prematura de las células del cumulus. En consecuencia, nuestros resultados parecían indicar que la mayoría de ovocitos recuperados de cabras prepúberes eran atrésicos, y que la atresia aumentaba con el diámetro ovocitario. Esta hipótesis parecía concordar con estudios previos, en el que se observó que los ovocitos recién recuperados de los folículos que ya habían reanudado la meiosis presentaban mayor incidencia de apoptosis que aquellos que todavía permanecían en parada meiótica (Warzych et al., 2006). Sin embargo, en el estudio realizado sobre apoptosis en cabras prepúberes observamos que los ovocitos que habían reanudado la meiosis en el momento de ser obtenidos del folículo presentaban menor incidencia de apoptosis que los que se encontraban todavía en parada meiótica, mientras que en ovocitos de vacas observamos lo contrario. Es posible que la dinámica de reanudación meiótica tras la liberación del folículo en ovocitos de cabras prepúberes sea mucho más rápida que en cabras adultas (Le Gal et al., 1992), que presentan el 100% de los ovocitos en estadío de vesícula germinal a las 3 h de la liberación del folículo. En cambio, en bovino, los ovocitos de menor tamaño que han reanudado la meiosis en el momento de ser liberados de los folículos son los que mayoritariamente provienen de folículos atrésicos.

Diversos estudios han descrito que los COCs que muestran signos tempranos de atresia dan lugar a un mayor porcentaje de blastocistos (Blondin y Sirard, 1995; Boni et al., 2002; de Wit et al., 2000; Nicholas et al., 2005), debido a que estos COCs sufren un proceso similar a la maduración dentro del folículo (Assey et al., 1994) que les proporciona mayor competencia para el desarrollo. Sin embargo, en cabras prepúberes el efecto de la atresia del COC en su capacidad para el desarrollo no había sido evaluado hasta el momento. Para ello, la incidencia de apoptosis en los ovocitos y las células del cumulus fue evaluada mediante dos técnicas complementarias: la tinción mediante Annexin-V, que permite distinguir las células en estadíos tempranos de apoptosis de las células en estadíos más tardíos; y la técnica de TUNEL, que permite observar las células que se encuentran en las últimas fases de la apoptosis. Estas técnicas fueron puestas a punto mediante la realización de un estudio previo en COCs bovinos, y posteriormente se aplicaron en COCs de cabras prepúberes. En general, pudimos observar que los COCs de mayor diámetro, que son más competentes para dar lugar a un embrión viable, son también los que sufren menor incidencia de apoptosis, tanto en vacas como en cabras prepúberes. La incidencia de apoptosis en ovocitos fue similar en las dos especies, según el tamaño y la técnica de detección utilizada. En ambos casos se pudo observar una reducción de la apoptosis ovocitaria durante la maduración *in vitro*, como había sido observada previamente en ovocitos humanos por Van Blerkom y Davis (1998). Sin embargo, estudios realizados en otras especies muestran un incremento de los niveles de apoptosis en ovocitos en cultivo (Fujino et al., 1996; Takase et al., 1995; Van Blerkom y Davis, 1998), que puede ser indicativo del posible efecto que los diferentes medios de cultivo pueden tener sobre estos ovocitos. En cabras prepúberes, al contrario de lo observado en otras especies, los COCs que no presentan ningún signo morfológico de atresia son los que dan lugar a un mayor porcentaje de blastocistos. La morfología del COC y el diámetro ovocitario en cabras prepúberes no parece afectar a la calidad de los blastocistos obtenidos, evaluada según el porcentaje de blastómeros apoptóticos (ACR), capacidad de eclosión y número de células del blastocisto. Curiosamente, el porcentaje de blastómeros

apoptóticos detectado en blastocistos de cabras prepúberes fue menor que el obtenido en blastocistos bovinos, aunque estos últimos estuvieron menos tiempo en cultivo. No obstante, los índices de apoptosis en los blastocistos de ambas especies se encuentran dentro de los índices que ya habían sido observados previamente en otros estudios (2.89 %, Warzych et al., 2006; 3-7 % Boelhauve et al., 2005; 4-9 %, Fabian et al., 2005; 10.3 %, Pomar et al., 2005; 12 %, Yuan et al., 2005; 9.8-14.2 %, Vandaele et al., 2006)

Teniendo en consideración todos los estudios realizados en esta tesis, podemos concluir que la capacidad de los ovocitos para llegar al estadío de blastocisto es mayor en ovocitos de mayor diámetro, con mayor actividad de MPF y menor incidencia de apoptosis, tanto a nivel ovocitario como en las células del cumulus. Parece ser que existen indicios de la existencia de una conexión entre la regulación de la maduración ovocitaria y la de la muerte celular, según diversos estudios realizados en invertebrados (*Drosophila*: Ivanovska et al., 2004; *starfish*: Sasaki y Chiba, 2004; Greenwood y Gautier, 2005; *Xenopus*: Smith et al., 2000; revisado por Greenwood y Gautier, 2005; *Caenorhabditis elegans*: Kritikou et al., 2006) y en mamíferos (ratón: Tatone et al., 2006; cerdo: Ma et al., 2005). Sin embargo, sería necesario realizar estudios complementarios para averiguar de qué modo se relacionan las dos rutas de regulación, y cual sería el efecto en la competencia del ovocito.

# 9. CONCLUSIONES

Las conclusiones de esta tesis fueron las siguientes:

- El diámetro del ovocito y la morfología del COC son buenos indicadores no invasivos de la competencia ovocitaria para producir embriones en cabras prepúberes. Así, para obtener el mayor porcentaje de blastocistos deben seleccionarse los COCs con un diámetro ovocitario superior a 135 µm y sin signos visibles de atresia.

- Los ovocitos de cabras prepúberes con una mayor competencia para el desarrollo también presentaron un mayor nivel de expresión de Ciclina B1-ARN en ovocitos inmaduros, una mayor acumulación de proteína $p34^{cdc2}$ y mayor actividad kinasa del *Maturation Promoting Factor* después de la maduración *in vitro*. Por lo tanto, estos parámetros pueden utilizarse como indicadores invasivos de calidad ovocitaria.

- En vacas y cabras prepúberes, el porcentaje de apoptosis en ovocitos y células del cumulus varía con el diámetro ovocitario. Además, una mayor incidencia de apoptosis en ovocitos y células del cumulus no afectó a la calidad de los blastocistos resultantes, evaluada según la incidencia de apoptosis en estos blastocistos.

- Las diferencias de morfología entre COCs sin signos y con signos visibles de atresia en cabras prepúberes se relaciona con un diferente grado de apoptosis en ovocitos y células del cumulus. La selección de COCs mediante su morfología antes de la MIV incrementa el porcentaje de blastocistos sólo en los ovocitos que han completado su crecimiento.

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