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Exploring the potential of prepubertal oocytes: a small ruminant model

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ABSTRACT

Juvenile *in vitro* embryo transfer (JIVET) employs oocytes from prepubertal females to reduce generation intervals. In human, it offers potential applications for fertility preservation in young cancer patients. Ruminant animals are good models for human research because of similar follicular dynamics and oocyte competence. Several studies have shown low embryo development of prepubertal oocytes. However, oocytes from follicles larger than 3 mm can develop into blastocysts at rates comparable to oocytes from adults. This review analyzes biomarkers of oocytes as a function of follicle size (larger or smaller than 3 mm). Follicular fluid from prepubertal females contains lower levels of n-3 fatty acids than that of adults; expression of epidermal growth factor receptor (EGFR) receptor in cumulus cells is higher in large than small follicles; the condensed germinal vesicle (GV) configuration is similar in prepubertal females to the adult ones, and prepubertal large follicles and oocytes are more susceptible to oxidative stress than adult ones. *In vitro* maturation (IVM) strategies have been investigated: capacitation IVM (CAPA-IVM) increases EGF receptor expression and promotes GV compaction; supplementation with bone morphogenetic protein 15 (BMP15) increases EGF receptor expression; and addition of antioxidants to IVM is essential. In conclusion, oocyte competence seems to be more related to follicular state than to female age, highlighting the importance of follicular selection and optimization of IVM conditions for improving JIVET outcomes.

Keywords: antioxidants, epidermal growth factor receptor, fatty acids, germinal vesicle, *in vitro* fertilization, JIVET, oocyte competence, prepubertal oocytes, small ruminants.

Introduction

In vitro embryo production (IVEP) is an assisted reproductive technology (ART) which aims to obtain embryos from immature oocytes. IVEP has many potential applications for animal husbandry as it enhances the genetic progress through the maternal lineage with females of high commercial value. Juvenile *in vitro* embryo transfer (JIVET) is an ART that enables the production of *in vitro* blastocysts from juvenile or prepubertal female oocytes. JIVET is of considerable interest in breeding programs due to its potential to accelerate genetic gain by reducing the generation interval. In livestock breeding, the integration of genomic selection with JIVET could profoundly influence the genetic architecture of animal populations.

In humans, protocols involving juvenile oocytes are primarily associated with fertility preservation in young patients undergoing oncological treatments. Conventional approaches for these patients include: (a) retrieving immature oocytes from small antral follicles, followed by *in vitro* maturation (IVM) and cryopreservation for future use; and (b) harvesting ovarian tissue, processing it into cortical strips, and performing ovarian tissue cryopreservation (OTC) for later transplantation and follicular stimulation. Both IVM-derived oocytes and oocytes obtained from successfully transplanted cryopreserved ovarian tissue have led to pregnancies and live births (reviewed by Anderson and Wallace 2011).

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Ruminants are considered a good model for human oocyte studies because they have similar follicular dynamics, a similar pattern of progressive oocyte competence acquisition before ovulation, and a similar timing of embryonic genome activation (reviewed by Sirard 2017). Moreover, small ruminants present the advantages of their low costs, ease of accommodation, great longevity, and easy handling. In livestock species several studies have been carried out using oocytes from prepubertal females with the general conclusion that the low blastocyst development obtained from them is due to the poor-quality oocytes obtained from these young ovaries (reviewed by Morton 2008; Pasquariello *et al.* 2024).

We define oocyte quality or oocyte competence as the ability to resume meiosis, reach the metaphase II (MII) stage, cleave correctly after fertilization, develop normally to the blastocyst stage, and bring healthy offspring to term. Oocyte competence is acquired progressively during folliculogenesis as the oocyte grows via gap-junction mediated communication with the cumulus cells. The positive and direct relationship among follicle size, oocyte diameter and oocyte competence for embryo development is well known (reviewed by Gandolfi *et al.* 2005).

At birth, ruminant females have ovaries with visible antral follicles. As they progress through puberty, both the size of the ovaries and the number of growing follicles increase. In sheep, Morton (2008) reported a significant increase in ovarian weight from birth to 8 weeks of age, with the number of growing ovarian follicles increasing significantly by 4 weeks of age. Therefore, the number of competent oocytes obtained per ovary depends on the number of size-selected antral follicles which can be related to the age of the prepubertal females.

For over 30 years, our laboratory has been working on IVEP in small ruminants, especially goats, using oocytes collected from 1 to 2 months old goats and 3 to 5 months old lambs which are the standard ages available at commercial abattoirs. These females are well before their puberty age, which occurs between 6 and 9 months of life (depending on the season of birth) and their ovaries are characterized by having small-diameter antral follicles. During these years, various media and culture strategies have been tested to increase the percentage of blastocysts obtained per ovary, but IVEP still shows highly variable results. This could be because the pool of oocytes obtained from the ovaries of slaughtered females is heterogeneous and of unknown developmental stage, as they come from follicles in different degrees of growth and atresia (reviewed by Paramio and Izquierdo 2014, 2016).

The search for adequate criteria to select competent oocytes before their use in IVEP programs is widely studied. The most common marker of oocyte competence is the size of the follicle and the oocyte. Other morphologic markers, such as cytoplasm appearance and the number of layers of cumulus cells are used to remove atretic oocytes. Even so, of these selected oocytes only 30–40% will produce blastocysts in cattle.In prepubertal oocytes, blastocyst output ranges from 5 to 20% (reviewed by Paramio and Izquierdo 2014).

For many years, the oocyte has been studied as the sole determinant of its ability to produce embryos, but currently, the characteristics of the elements surrounding the oocyte, such as the cumulus cells, the follicular fluid and their effect on the dynamics of meiosis resumption are being studied as markers of oocyte competence.

Oocyte competence and follicular fluid composition changes throughout follicle growth and development. Follicular fluid has the potential as a biomarker for oocyte quality as it reflects the oocyte's *in vivo* microenvironment. One of the principal follicular fluid compounds affecting oocyte competence is fatty acids, due to their role in energy production (reviewed by Warzych and Lipinska 2020). Specific fatty acids and their concentrations within the follicle are related to female fertility in various species, including humans (reviewed by de Andrade Melo-Sterza and Poehland 2021).

The somatic cells surrounding the oocytes could be good biomarkers of oocytes' competence. Cumulus cells deliver nutrients and regulatory factors to the oocyte for its growth and maturation, and in turn the oocyte controls the differentiation and function of the cumulus cells by releasing potent paracrine growth factors, commonly referred to as oocyte-secreted factors (OSF). Major OSFs that regulate oocyte developmental competence are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). These OSF, together with FSH, control the progressive acquisition of epidermal growth factor (EGF) responsiveness of cumulus cells as folliculogenesis progresses. EGF signaling network induces oocyte nuclear and cytoplasmic maturation and cumulus expansion after the LH pre-ovulatory surge (reviewed by Richani and Gilchrist 2018). The failure of low developmental competence oocytes to respond to EGF is due to the immaturity of EGF receptor (EGFR) in the cumulus cells (Procházka *et al.* 2000; Ritter *et al.* 2015). The study of the EGFR expression in cumulus cells could help us to understand the prepubertal oocytes competence.

Oocyte competence involves progressive germinal vesicle (GV) chromatin condensation related to a decrease in transcriptional activity (reviewed by Luciano and Lodde 2013) in preparation for meiosis resumption. In cattle, it has been suggested that a specific GV chromatin configuration could be a good indicator of oocyte competence (reviewed by Sirard 2019).

In IVEP programs, oocytes spontaneously resume meiotic maturation when removed from the follicle and placed *in vitro*. The oocytes liberated from the follicle are exposed to O_2 rich atmosphere, which increases intracytoplasmic reactive oxygen species (ROS) and is harmful to oocyte competence. Inside the follicle, the oocytes are protected by antioxidant compounds (reviewed by Guérin *et al.* 2001). In mice, it has been shown that prepubertal oocytes are particularly sensitive to ROS due to their reduced antioxidant

capacity, as assessed by intracytoplasmic glutathione (GSH) levels (Jiao *et al.* 2013).

This review aims to describe key characteristics of prepubertal small ruminant oocytes and identify specific parameters that may serve as markers of oocyte competence. These include: (a) the composition of fatty acids in follicular fluid, (b) EGFR expression in cumulus cells, (c) germinal vesicle (GV) chromatin configuration, and (d) oxidative stress levels in prepubertal goat oocytes. In addition, we outline *in vitro* maturation (IVM) strategies designed to address deficiencies in these parameters and enhance the developmental competence of prepubertal oocytes. These strategies include: (1) evaluating the effects of supplementing the IVM medium with fatty acids, (2) using oocyte-secreted factors (OSFs) as IVM supplementation to enhance the EGF signaling network, (3) applying a biphasic IVM system (CAPA-IVM) to delay spontaneous meiotic resumption and maintain cumulus-oocyte communication, and (4) incorporating various antioxidants into the maturation medium to mitigate oxidative stress in prepubertal goat oocytes.

Characteristics of oocytes from prepubertal females

In small ruminants, Crozet *et al.* (1993) and Cognie *et al.* (1995) reported the birth of the first kid and lamb born from IVM-IVF and *in vitro* culture (IVC) oocytes of adult females, respectively. In terms of efficiency, in small ruminants approximately 75–90% of immature oocytes undergo nuclear maturation and reach the MII stage, 50–80% of them undergo fertilization and cleave into 2-cell embryos, but only 20–50% reach the blastocyst stage (reviewed by Souza-Fabjan *et al.* 2023). In prepubertal females, nuclear maturation is similarly high, however, blastocyst rates are lower, ranging from 5% to 20% (reviewed by Paramio and Izquierdo 2014). Fig. 1 shows the conventional protocols for *in vitro* embryo production of prepubertal goats.

Ovaries and follicles of prepubertal females

Ovaries from prepubertal goats are characterized by a smooth texture and a high number of small antral follicles. Evaluation of different oocyte collection techniques revealed the numbers of oocytes selected per ovary were 1.71, 1.27 and 6.05 for dissection, aspiration and slicing, respectively (Martino *et al.* 1994*a*, 1994*b*). The slicing technique was selected for oocyte collection from this kind of ovaries because of the difficulty in aspirating these small and soft follicles. There was a positive relationship between the oocyte, the follicle diameter and the meiotic competence. Oocytes coming from follicles larger than 2.5 mm were meiotically competent but with high IVF anomalies. These anomalies were significantly reduced in oocytes recovered from follicles larger than 3 mm (Martino *et al.* 1994*a*, 1994*b*). In the case of prepubertal goats, most follicles are smaller than 2.5 mm, with only 1.1 follicles larger than 3 mm per ovary. In these studies, the conclusion was that oocytes from follicles larger than 3 mm presented higher normal fertilization. In lamb oocytes, Morton (2008) suggested that ovary weight and the number of growing follicles increased significantly by 4 weeks of age and remained constant until 33 weeks of age. Despite the high number of follicles, these oocytes displayed reduced embryo development. Gonadotropin-primed ovarian stimulation of 1.5–3 months old goats resulted in recovery of 15–35 oocytes per female, with an average of 22 oocytes suitable for IVM, which was higher than in adult goats and similar embryo development in both cases (reviewed by Tibary *et al.* 2005). Early testing of different IVM media (Mogas *et al.* 1997*a*) and different *in vitro* embryo culture media (Izquierdo *et al.* 1999) in prepubertal goats showed low blastocyst development. This low embryo development rate observed in these studies

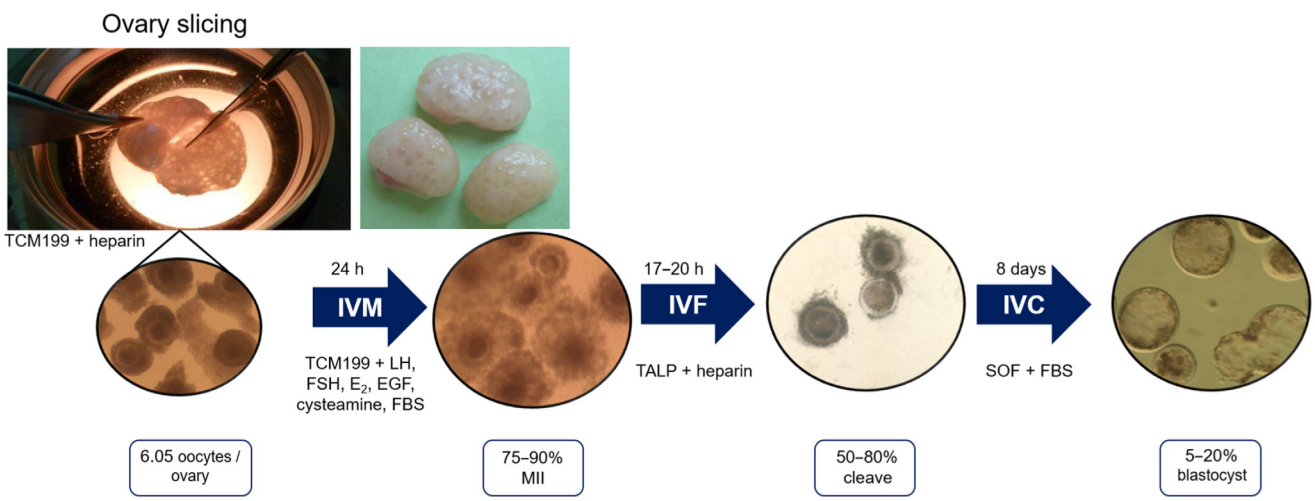

Fig. 1. Protocols for *in vitro* embryo production of prepubertal goats.

was caused by a high percentage of abnormal zygotes after IVF. These anomalies were polyspermy, polygyny and nuclei asynchrony due to a delay or failure in the decondensation of the male pronucleus (Mogas *et al.* 1997*a*, 1997*b*). After IVF, cleavage oocytes of 2–4 cells showed that 59% of these embryos were haploid and 1% triploid (Villamediana *et al.* 2001).

In humans, results showed follicle size is predictive of oocyte developmental competence, including oocyte maturity (follicle >15 mm), normal fertilization (>16 mm) and embryo development (>15 mm) (Zheng *et al.* 2025). However, in relatively small follicles of around just a few millimeters in size, an oocyte diameter reaches its final dimension of 110–120 μm on average and does not appear to increase in size (or only marginally) during follicular development until ovulation (Pors *et al.* 2022).

Structural characteristics of prepubertal oocytes

Oocytes from juvenile females are less capable of producing embryos and offspring to term compared to their adult counterparts (reviewed by Armstrong 2001). There are structural and metabolic differences between adult and prepubertal oocytes. Compared to adult cows, oocytes from calves are smaller, metabolize less glutamine and pyruvate, have lower protein synthesis (Gandolfi *et al.* 1998), less mitochondria (de Paz *et al.* 2001) and store less maternal mRNA and proteins (Romar *et al.* 2011). In addition, adult cow oocytes present upregulation of genes related to mitochondrial activity, cell differentiation and transcription control, whereas calf oocytes have upregulation of genes related to apoptosis (Dorji *et al.* 2012). Oocytes from prepubertal goats also show ultrastructural and functional deficiencies such as impaired distribution of cortical granules (Velilla *et al.* 2004) and mitochondria (Velilla *et al.* 2006), disorganized microtubules and microfilaments (Velilla *et al.* 2005), altered total RNA content, p34 and cyclin B1 expression, and activity of the maturation promoting factor (MPF) (Anguita *et al.* 2007). Based on scanning electron microscopy analysis of the *zona pellucida* (ZP) configuration, Villamediana *et al.* (1999) concluded that the IVM process results in adequate and similar development of the ZP surface in oocytes from adult and prepubertal goats.

Blastocyst development according to follicle and oocyte growth

Crozet *et al.* (1995) described a specific relationship between follicle size and oocyte competence in adult goats. Thus, a blastocyst percentage of 6%, 12%, 26% and 41% was obtained from oocytes recovered from follicles 2–3 mm, 3.1–5 mm, >5 mm in diameter and ovulated oocytes, respectively. In sheep, Cognie *et al.* (1998) also achieved a higher percentage of blastocysts in oocytes from follicles >3.5 mm (40%) compared with those <3.5 mm (26%). Studying prepubertal goat oocytes and classifying oocytes coming from follicles smaller and larger than 3 mm, significant differences were found between blastocyst yields of oocytes recovered from follicles <3 mm of prepubertal goats compared to oocytes from adult goats (5.4% and 20.8%, respectively). However, these differences between prepubertal and adult goats disappeared if their oocytes were recovered from large follicles (18.1%) (Romaguera *et al.* 2011). In lamb oocytes, blastocyst development was higher in oocytes recovered from follicles ≥3 mm compared to those from smaller follicles (11.1% and 6.5%, respectively) (Contreras-Solís *et al.* 2021). In calf oocytes, Kauffold *et al.* (2005) reported higher blastocyst yield in oocytes from follicles larger than 8 mm (47%) than from smaller follicles (<15%), but found no differences between calf and cow oocytes from large follicles (~59%).

Slicing the ovary is the conventional method to release oocytes from prepubertal ovaries, and these oocytes must be selected based on their morphological characteristics. To study the relationship between oocyte and embryo development, prepubertal goat oocytes were classified into four categories based on their diameter: <110 μm, 110–125 μm, 125–135 μm, and >135 μm. Anguita *et al.* (2007) observed blastocyst rates after IVF of 0%, 0%, 1.9%, and 12.5% for each oocyte category. Moreover, the oocyte diameter was also positively related to the amount of cyclin dependent Kinase1 (CDK1) protein and MPF activity in prepubertal females.

Blastocyst development according to the Brillant Cresyl blue selection

Measuring oocyte diameter is a time-consuming procedure during which oocytes are exposed to light and harmful atmospheric effects. Brilliant Cresyl Blue (BCB) stain is known to be a non-invasive methodology that allows selection of oocytes with larger diameters from a heterogeneous pool recovered by slicing from small ovaries. The BCB test determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH), a pentose phosphate pathway enzyme that gradually decreases its activity as oocytes reach their growth phase. BCB dye can be reduced by G6PDH activity, therefore oocytes that have completed their growth phase cannot reduce BCB to a colorless compound and exhibit a blue-colored cytoplasm (BCB+).

Our first study using this method was on prepubertal cattle oocytes (Pujol *et al.* 2004).We observed an increase in the percentages of the blastocyst yield of BCB+ compared to BCB- oocytes (12.3% and 1.6%, respectively). In prepubertal goats, Rodríguez-González *et al.* (2002) found that the mean diameter of BCB+ oocytes (136 μm) was larger than BCB- oocytes (125 μm). In comparison to BCB- oocytes, BCB+ oocytes presented higher normal fertilization (zygotes with two pronuclei) (39.7% and 21.0%, respectively), blastocyst development (8 and 2%, respectively) and higher blastocyst quality assessed by the number of blastocyst cells. The percentage of stained BCB+ oocytes was 28.6% related to

the total oocytes selected by conventional morphological characteristics which means the important limitations of the morphological selection.

In prepubertal sheep, BCB+ oocytes were also larger (123 μm) than BCB- (106 μm), showing a higher percentage of normal fertilized zygotes (40% and 20%, respectively) and higher blastocyst development after IVF (21% and 9%, respectively) (Catalá *et al.* 2011). Moreover, BCB+ oocytes presented higher mitochondrial and MPF activity, and produced blastocysts with higher numbers of cells than BCB- oocytes. Mitochondrial distribution and mRNA expression of *ATP1A1*, *COX1*, *CPEB* and S100A10 were not affected by oocyte BCB selection. In lamb oocytes recovered by slicing, the percentage of BCB+ oocytes was low (20%).

Blastocyst development after intracytoplasmic sperm injection

The development of intracytoplasmic sperm injection (ICSI) technique has been used for low-quality oocytes of humans. In animals, the use of ICSI has been specially developed in some species where poor results are obtained with conventional IVF due to polyspermy, such as in pigs, or due to the impossibility to obtain repeatable results, such as in horses. In addition, ICSI could also be useful in the evaluation of oocyte competence because it reduces the variation in sperm penetration and allows potential fertilization and embryo development of each one of the MII-oocytes injected.

We suggest that ICSI could have improved embryo development of prepubertal oocytes due to an accurate selection of MII-oocytes before sperm injection and thanks to a lack of polyspermic zygote formation. Goat prepubertal IVM-oocytes were allocated into four groups depending on their diameter (Group A: <110 μm; Group B: 110–125 μm; Group C: 125–135 μm; Group D: >135 μm), fertilized by ICSI and cultured for 192 h. Results showed a positive correlation between oocyte diameter and embryo development (blastocysts: Group A: 0%; Group B: 0%; Group C: 15.9%, and Group D: 11.1%) (Jiménez-Macedo *et al.* 2006). In a parallel study using the same classification sizes, blastocyst development after IVF was significantly higher in D category than in C (Anguita *et al.* 2007). The same trend was observed in lamb oocytes. BCB+ oocytes did not present differences in blastocyst development compared to BCB- oocytes after ICSI (18.6% and 14.8%, respectively). But after IVF, blastocyst development was significantly higher for BCB+ oocytes than for BCB- oocytes (31.7% and 6.7%, respectively) (Catalá *et al.* 2012).

The use of ICSI in prepubertal goats was previously carried out by Jiménez-Macedo *et al.* (2005) using chemical activation after the injection (ionomycin for 5 minutes plus 6-dimethylaminopurine (6-DMAP) for 4 h). In this study, the percentage of embryos obtained and developed beyond the 8-cell stage was significantly higher for ICSI than for IVF. However, a high number of parthenogenic embryos caused by chemical activation was observed. Later, Jiménez-Macedo *et al.* (2006) achieved 11% of blastocysts using heparin plus ionomycin to capacitate the fresh sperm sample to avoid posterior chemical activation before injection. Using frozen-thawed semen, similar blastocyst development for ICSI, IVF and parthenogenic activation (PA) (13.1%, 13.6%, and 13.5%, respectively) was observed in lambs (Catalá *et al.* 2012). After sperm injection, lamb oocytes needed a short activation of 4 minutes with ionomycin.

Afterwards, Menéndez-Blanco *et al.* (2019) tested different protocols of ICSI in prepubertal goat oocytes and achieved 12.1% and 18.5% of blastocysts from frozen-thawed and fresh semen, respectively. This improvement of blastocyst development from prepubertal goat oocytes could be due to a better selection of spermatozoa by using the hyaluronic acid (HA)-binding method. This system of sperm selection (HA-binding) allows for the selection of spermatozoa with higher chromatin condensation and higher nuclear maturation that are attached to HA (Jakab *et al.* 2005; Parmegiani *et al.* 2012). These experiments in prepubertal goat oocytes did not use chemical activation after sperm injection. Moreover, increased blastocyst production was observed by supplementing the IVM media with an antioxidant (crocetin) and reducing the cytoplasmic ROS, because ICSI is a procedure which exposes the gametes to a high oxygen (O_2) atmosphere (Menéndez-Blanco *et al.* 2020*a*).

Response to cryopreservation of oocytes and embryos of prepubertal females

Oocyte cryopreservation

The cryopreservation of prepubertal oocytes in small ruminants represents an important development in reproductive biotechnology, with clear applications in both research and animal breeding. Long-term storage of oocytes is valuable for livestock and human fertility programs. Vitrification has emerged as the preferred cryopreservation technique for oocytes because it avoids ice crystal formation through very rapid cooling. However, its application in small ruminants is still limited. These oocytes are more sensitive to the physical and chemical stresses of vitrification, including exposure to high concentrations of cryoprotectants (CPAs) and osmotic and chilling injuries which impair their developmental competence. In addition, the lack of standardized protocols makes it difficult to compare results between studies (reviewed by Souza-Fabjan *et al.* 2023).

Information regarding oocytes cryopreservation in sheep and goat is relatively limited compared to that in cows. Attempts to apply conventional slow freezing techniques in these small ruminants have consistently resulted in low embryonic developmental outcomes (Le Gal 1996; Bhat *et al.* 2014). Consequently, most studies have focused on adapting vitrification protocols from other species in an effort to minimize cryoinjuries. Despite these efforts, the developmental competence of vitrified ovine (Hosseini *et al.* 2015; Quan *et al.* 2016) or caprine (Purohit *et al.* 2012) immature or *in vitro*

matured oocytes remains low, with reported blastocyst rates ranging from 0% to 12.5%.

Succu *et al.* (2021) provides a standardized vitrification protocol specifically designed for *in vitro* matured ovine oocytes obtained from both prepubertal (30–40 days of age) and adult females. They observed that prepubertal sheep oocytes have lower cryotolerance than adult oocytes, with reduced post-warming survival and membrane integrity. Trehalose supplementation during *in vitro* maturation improved survival rates of prepubertal oocytes to levels comparable with adults but did not enhance fertilization or embryo development. Interestingly, using low-calcium vitrification media increased fertilization rates in prepubertal oocytes, though embryo yield remained unchanged. The post-warming recovery period also proved critical: while a 4-h post-warming culture optimized developmental competence in adult oocytes, juvenile oocytes showed peak mitochondrial activity at 4 h but increased ROS levels and spontaneous activation beyond 2 h. Despite these optimizations, their developmental competence remains inferior to that of adult counterparts. Similar findings were reported in goats by Menéndez-Blanco *et al.* (2020*b*), who assessed the effects of vitrification on *in vitro* matured oocytes collected from prepubertal goats (1–2 months old). Vitrification increased intracellular ROS levels and reduced viability, with a significant rise in the proportion of dead oocytes. Following ICSI, fertilization and cleavage rates were not significantly affected, but blastocyst development was compromised. After parthenogenetic activation, vitrified oocytes showed significantly lower cleavage and blastocyst rates compared to non-vitrified controls.

Vitrification of adult bovine oocytes has been extensively studied, resulting in several methodological advances aimed at improving cryosurvival and developmental competence (reviewed by Mogas *et al.* 2024). In contrast, studies on the vitrification of prepubertal bovine oocytes are still limited, and their intrinsic features, such as incomplete cytoplasmic maturation, altered spindle stability, and higher sensitivity to oxidative and osmotic stress, make the development of efficient cryopreservation protocols particularly challenging. Albarracín *et al.* (2005) reported that vitrification using open pulled straw (OPS) method caused significant cytoskeletal alterations in both prepubertal and adult bovine *in vitro* matured oocytes. Spindle morphology and actin microfilament organization was more severely affected in prepubertal oocytes after vitrification, whereas adult oocytes showed greater sensitivity to CPA exposure. Developmental competence, particularly blastocyst formation, was lower in prepubertal oocytes following vitrification. Sprícigo *et al.* (2017) extended this work by evaluating whether supplementation of the maturation medium with L-carnitine and resveratrol could enhance the cryotolerance of prepubertal *in vitro* matured oocytes. These antioxidants partially preserved spindle structure, reduced apoptosis markers, and maintained gene expression profiles associated with oxidative stress resistance. Despite these cytological and molecular improvements, the enhancements did not translate into significant increases in cleavage or blastocyst rates, indicating that while antioxidant supplementation can mitigate some cryodamage, it does not fully overcome the developmental limitations of vitrified prepubertal bovine oocytes.

Embryo cryopreservation

The cryopreservation of *in vitro* produced (IVP) embryos from prepubertal goats or sheep enables long-term storage and offers flexibility for embryo transfer scheduling.In general, it facilitates safe international movement of livestock genetics without transporting live animals. Studies on cryopreservation of IVP embryos from ovine and caprine species highlight a complex interplay of the age of the donor, developmental embryo kinetics, and embryo stage at vitrification. In caprine species, Leoni *et al.* (2009) demonstrated that *in vitro* produced embryos, derived from oocytes collected from FSH-stimulated prepubertal goats via laparoscopic ovum pick up (LOPU), showed significantly reduced re-expansion rates following vitrification and warming when compared to those derived from adult donors (40.3% and 62.5%, respectively). This highlights the reduced cryotolerance of embryos from prepubertal oocytes. In contrast, Morató *et al.* (2011) found no differences in survival rate between embryos from prepubertal goats (51.8%) and those from adult goats (40.7%). In addition, when analyzing what impact the embryo's stage at vitrification has, they found that unlike adult goats, which showed markedly improved survival when vitrified and then expanded, hatching-stage or hatched embryos derived from prepubertal oocytes showed similar survival rates across all developmental stages, with a slight advantage observed in the hatching-stage group. Interestingly, when comparing embryos from prepubertal and adult donors at the same developmental stage, survival after warming was only higher in non-expanded blastocysts from prepubertal females. These results suggest that, unlike adult embryos, the developmental stage at vitrification is less critical for prepubertal goat blastocysts, suggesting that embryos from younger donors may respond differently to the stresses associated with cryopreservation.

Leoni *et al.* (2006) investigated the cryotolerance of *in vitro* produced ovine embryos from prepubertal and adult donors, focusing specifically on post-warming survival. Their results demonstrated that blastocysts originating from prepubertal sheep exhibited significantly lower re-expansion and hatching rates after warming compared to those from adults. Furthermore, the timing of blastocyst formation played a critical role: embryos that reached the blastocyst stage earlier showed higher survival rates following vitrification, regardless of donor age. In contrast, late-forming blastocysts, particularly from prepubertal donors, had markedly reduced viability after warming. These findings highlight that both the developmental speed and the donor's age substantially influence the success of vitrification in ovine *in vitro* produced embryos.

Markers of oocyte competence in prepubertal females and IVM strategies to improve them

Follicular fluid fatty acid composition

Composition of follicular fluid can provide useful information about the stage of the follicle. Owing to the close and physical relation between oocyte and follicular fluid, the composition of this fluid can be determinant of oocyte quality (Dumesic *et al.* 2015). One of the follicular fluid compounds affecting oocyte competence are fatty acids. Fatty acids are an important component of the follicular fluid because they are a considerable source of energy for growing and developing the oocytes (reviewd by McKeegan and Sturmey 2012). Fatty acids have the capacity to generate 5–6-fold more energy than glucose, which means that this form of metabolism for generating ATP is highly efficient. Specific fatty acids and their concentrations inside of the follicle are related to female fertility in different species including humans (reviewed by Warzych and Lipinska 2020). Polyunsaturated fatty acids (PUFAs), including n-6 linoleic acid (LA) and n-3 linolenic acid (ALA) play an important role in reproductive physiology, affecting oocyte quality (Wathes *et al.* 2007).

In cattle, Sinclair *et al.* (2008) concluded that increasing levels of palmitic and stearic acids in follicular fluid were associated with morphologically poor and good cumulus-oocyte complexes (COC), respectively. Matoba *et al.* (2014) concluded that oocytes with higher blastocyst development presented higher concentrations of ALA, and lower concentrations of palmitic acid and total saturated fatty acids (SFA). In a different follicular fluid study of women who were following IVF treatment, O'Gorman *et al.* (2013) concluded that fatty acids in follicular fluid could serve as potential biomarkers for oocyte developmental competence, as they found differences in follicular fluid composition of the fatty acids in oocytes that cleaved compared to the ones that did not after fertilization. Palmitic acid, total saturated fatty acids (SFA) and n-6 to n-3 PUFA ratio were significantly higher in the non-cleaved group, whilst stearic and arachidonic acids, total PUFA and total n-3 PUFA were increased in the cleaved group. In goats, assessing follicular fluid of adult and prepubertal females, the percentages of ALA, total n-3 PUFAs and total SFAs were significantly higher in adults; and the percentages of LA, total n-6 PUFAs, total PUFAs and total monounsaturated fatty acids (MUFAs) were higher in prepubertal females. Thus, the ratio n-6 to n-3 PUFAs was higher in prepubertal females compared to their adult counterparts (6.3 and 2.6, respectively) (Izquierdo *et al.* 2022). Fig. 2 represents follicular fluid fatty acids composition of prepubertal and adult goats. In cattle, Homa and Brown (1992) showed that LA represented about a third of the total fatty acids but this LA proportion was higher in small follicles (1 to 3 mm) and ALA was higher in large follicles (>7 mm). In prepubertal goats, the proportion of SFA, MUFAs

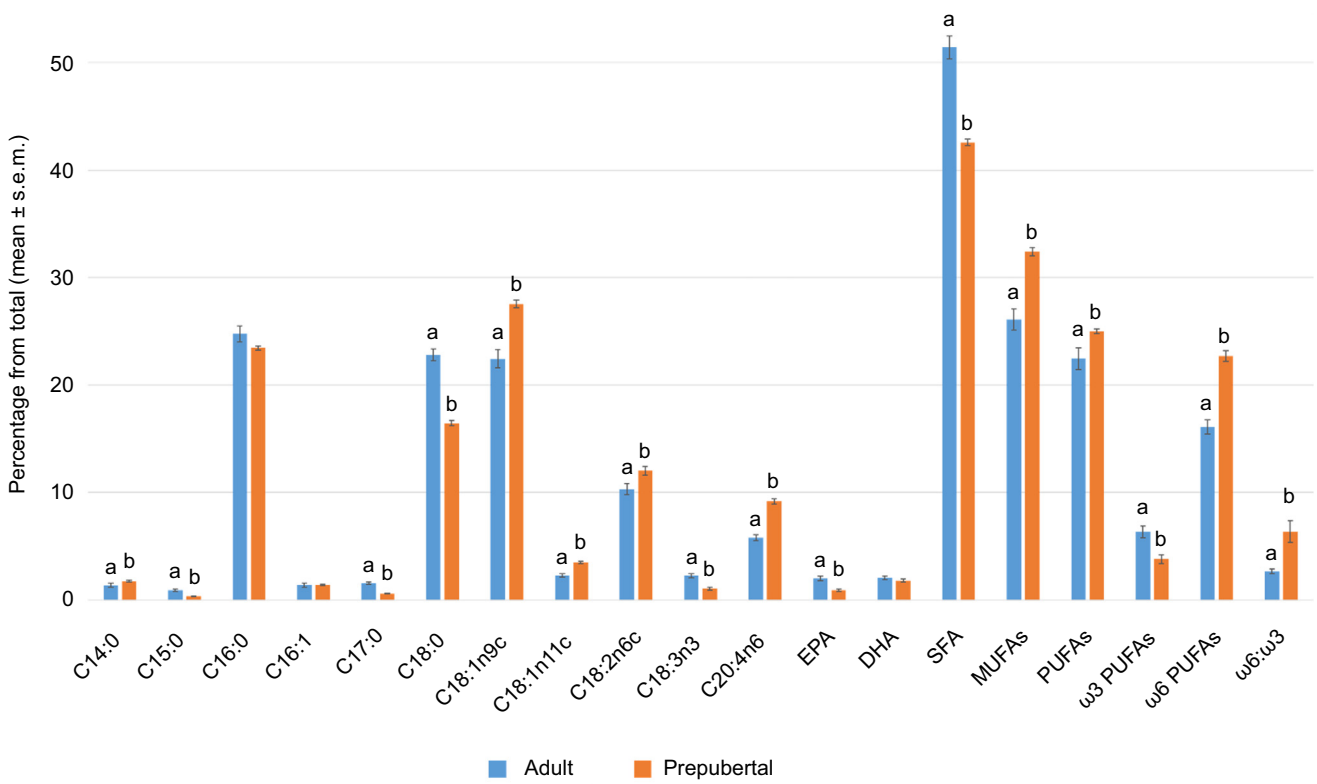

Fig. 2. Follicular fluid fatty acids composition of prepubertal and adult goats.

and PUFAs was significantly higher in small follicles than in large ones (Izquierdo *et al.* 2022). PUFAs, including n-6 and n-3, are essential fatty acids but cannot be synthesized in the body and must be provided from diet. In cattle, females which have received a diet supplementation with 1% dry matter of n-3 PUFAs, their follicular fluid was 1.2-fold higher in n-3 PUFAs and their oocytes developed higher quality blastocysts compared to control animals (Freret *et al.* 2019). The authors concluded that this supplementation could benefit the *in vitro* embryo production program. In conclusion, it seems that competent oocytes are related to the n-3 PUFA concentrations in their follicular fluid.

Supplementation of IVM medium with fatty acids

Oocytes accumulate lipid droplets in their cytoplasm during follicular development (McEvoy *et al.* 2000). The color of the ooplasm reflects the amount of fatty acids stored. In cows, oocytes with dark cytoplasm have more lipids (Leroy *et al.* 2005) and more mitochondria (Jeong *et al.* 2009) than oocytes with pale cytoplasm, which results in better developmental competence. The content of these fatty acids is reduced during maturation and blastocyst development (Sturmey and Leese 2003), highlighting the importance of the use of fatty acids for energy provision and oocyte quality (reviewed by Dunning *et al.* 2014). Fatty acid oxidation through β-oxidation is necessary for oocyte development. If β-oxidation is enhanced by adding fatty acids to IVM media, oocyte developmental capacity is increased in mice (Dunning *et al.* 2010), pig (Somfai *et al.* 2011), lamb (Reader *et al.* 2015) and cow (Sutton-McDowall *et al.* 2012; Ghanem *et al.* 2014). In cattle, Marei *et al.* (2009) reported positive effects on oocyte maturation, embryo development and embryo quality with the addition of 50 μM of ALA, but addition of 200 μM ALA (supraphysiological concentration) had a detrimental effect on the oocyte nuclear maturation, characterized by an extrusion of an abnormal second polar body, which may indicate hypermaturation/ageing of the oocytes. The addition of LA to IVM medium has also shown a negative effect on oocyte nuclear maturation and embryo development at physiological concentrations of 50, 100 and 200 μM (Marei *et al.* 2010). Similarly, Khalil *et al.* (2013) reported the negative effects of 100 μM LA concentration on IVM media, but these effects were alleviated by the addition of antioxidants such as vitamin E and glutathione peroxidase.

In prepubertal lamb oocytes, the addition of 50, 100, and 200 μM of ALA to the IVM medium was tested for impact on embryo development. No differences were observed in blastocyst yield among 50, 100, and 200 μM ALA groups (6.9%, 11.5% and 14.0%, respectively) and medium without ALA (12.0%) but the total cell number were significantly improved when using 50 μM ALA compared with the other groups (Ghaffarilaleh *et al.* 2014). In prepubertal goat follicular fluid, investigated LA concentration ranged from 247 μM to 319 μM and ALA concentration ranged from 8.39 μM to 41.19 μM, both without effect on follicle diameter (Roura *et al.* 2018).

In prepubertal goat oocytes, the effect of LA and ALA addition to IVM media on blastocyst production was tested in different concentration ratios (LA:ALA 50:50, 100:50 and 200:50 μM) (Roura *et al.* 2018). No differences were found among experimental groups when nuclear maturation (MII) was assessed, as it was nearly 90% in all the groups. Blastocyst production from the different groups was: 22.3% for fetal calf serum control group, 22.6% for LA:ALA 50:50 μM treatment group, 21.0% for LA:ALA 100:50 μM and 9.6% for 200:50 μM treatment groups. No statistically significant differences were found. Nevertheless, oocytes matured with 200:50 μM LA:ALA ratio in IVM medium had significantly higher rates of polyspermy (58.2%), lower normal fertilization and lower blastocyst development compared with other groups and the control group. However, we did not find any differences in ATP levels between the treatment groups. In adult goats, the addition of 50 μM of ALA to the IVM medium significantly increased the percentage of blastocysts after IVF and PA (Veshkini *et al.* 2016). This lack of effects of ALA in prepubertal sheep and goat oocytes could be explained by the different response of prepubertal oocytes compared to oocytes from adult females.

Epidermal growth factor receptor expression

Acquisition of oocyte competence also depends on the intricate bi-directional communication axis between the oocyte and follicular somatic cells- of the COC. Richani and Gilchrist (2018) proposed that EGF signaling is a key regulator of oocyte competence acquisition during the antral phase of folliculogenesis. EGFR mediates communication about regulating maternal mRNA translation between cumulus cells and the oocyte. COCs from small antral follicles have an underdeveloped EGFR signaling system and cannot respond to EGF cues as a means of restricting the ovulatory signal on them (Procházka *et al.* 2000; Prochazka *et al.* 2003). It develops, at least in part, by the FSH that stimulates follicular growth and oocyte cues through OSFs (Sugimura *et al.* 2015). OSFs drive the cumulus cells capacity to recognize the LH-EGF ovulatory cascade for oocyte maturation and cumulus expansion by promoting the expression of the EGFR (Su *et al.* 2010; Ritter *et al.* 2015).

During mouse folliculogenesis, the expression of EGFR protein and mRNA showed a marked increase between the preantral granulosa cells and the cumulus cells from early antral follicles (El-Hayek *et al.* 2014). A study in pigs found similar amounts of EGFR mRNA in COCs from follicles larger or smaller than 4 mm but higher amounts of the EGFR protein in the larger follicles (Ritter *et al.* 2015). In contrast, another study found that cumulus cells from small (1–2 mm), medium (3–4 mm) and large follicles (6–7 mm) contained similar amounts of the EGFR protein (Prochazka *et al.* 2003), but only EGFR from the 6–7 mm follicles were able to be extensively phosphorylated in response to EGF.

The COCs of prepubertal females are characterized by a low number of cumulus cells. In prepubertal mouse oocytes, an average of 1.61 layers of cumulus cells surrounded oocytes in follicles from the youngest cohort, whereas this gradually increased to 3.92 layers of cumulus cells per oocyte in follicles from the oldest cohort (Kusuhara *et al.* 2021). In COCs of prepubertal goat and sheep, pools of 70 COCs were necessary to retrieve enough cumulus cells for a western blot analysis of EGFR expression at the time of follicular recovery. Results showed that large follicles (≥3 mm) had a higher EGFR expression than the ones from small follicles (<3 mm), both in goats (1.87-fold difference) and sheep (2.34-fold difference) (Ferrer-Roda *et al.* 2024*a*). A sample of cumulus cells from adult oocytes showed a 1.82-fold increase in value compared to prepubertal small follicles for goats and a 2.25-fold increase for sheep, which is a value similar to that of prepubertal large follicles for each species. In conclusion, expression of EGFR in cumulus cells was related to the follicle size from which the oocytes were recovered, regardless of female age.

An approach to increase the expression of EGFR in cumulus cells of prepubertal goat oocytes involved using a biphasic maturation protocol (CAPA-IVM). EGFR protein levels were quantified in cumulus cells after CAPA-IVM and conventional IVM. The expression of EGFR in cumulus cells from large follicles was higher in CAPA-IVM compared to conventional IVM. However, in small follicles, EGFR expression decreased in CAPA-IVM compared to the control, indicating a lack of response in these small follicles due to their high immaturity (Ferrer-Roda *et al.* 2024*b*).

In humans, where approximately 99% of follicles undergo atresia rather than reaching full maturation and ovulation, OSFs may play a crucial role in promoting follicular dominance and supporting the selection of the ovulatory follicle (reviewed by Anderson and Pankhurst 2025).

Supplementation of IVM medium with exogenous oocyte-secreted factors

OSFs, such as BMP15 and GDF9, are soluble paracrine factors that drive cumulus cell differentiation and increase the amount of EGFR expression. Numerous studies have shown that COCs co-cultured with native OSF from denuded oocytes have increased oocyte developmental competence in cattle (Hussein *et al.* 2006), mice (Sudiman *et al.* 2014*a*), pigs (Gomez *et al.* 2012) and goats (Romaguera *et al.* 2010). Recombinant exogenous BMP15 and GDF9, however, show species-specific differences in their success at improving blastocyst production when added to IVM. While GDF9 is more significant in poly-ovulatory species like mice (Dong *et al.* 1996; Yeo *et al.* 2008), BMP15 is more effective than GDF9 in enhancing the developmental competence of mono-ovulatory species like cattle (Hussein *et al.* 2006; Sudiman *et al.* 2014*b*; Sugimura *et al.* 2014). These OSFs promote cumulus cells proliferation (Gilchrist *et al.* 2006), glucose metabolism (Sugiura *et al.* 2007), the generation of new transzonal projections (TZP) (El-Hayek *et al.* 2018), and the acquisition of responsiveness to EGF signals (Su *et al.* 2010; Ritter *et al.* 2015).

The addition of OSFs to the IVM medium showed that GDF9 did not affect embryo development in adult bovine oocytes but BMP15 improved blastocyst production and their quality (Hussein *et al.* 2006; Sudiman *et al.* 2014*b*; Sugimura *et al.* 2014). In sheep, GDF9 did not improve blastocyst formation but it increased blastocyst quality (Varnosfaderani *et al.* 2013). In porcine (Sugimura *et al.* 2015) and buffalo (Gupta *et al.* 2017), the blastocyst production was increased when the two OSFs were combined, but each one alone was unable to improve blastocyst formation.

To test their effect on prepubertal goat oocytes, IVM was performed in the presence of BMP15 (100 ng/mL) and GDF9 (100 ng/mL), alone or in combination (Ferrer-Roda *et al.* 2025). Thus, the results showed that the addition of BMP15 enhanced cumulus-oocyte communication (assessed by TZP density), cumulus expansion, EGFR expression on cumulus cells and blastocyst development after parthenogenic activation. IVM supplementation with GDF9 did not improve results from the control group in any parameters studied. Moreover, GDF9 in combination with BMP15 only improved cumulus expansion over control group. The lack of effect when both OSFs were combined could indicate an antagonist effect of GDF9 over BMP15, or a harmful effect of the high OSF concentrations. In a previous study from our laboratory, we hypothesized that in prepubertal goats GDF9 is not involved in the improvement of oocyte competence caused by native OSFs (Romaguera *et al.* 2010). In that study, co-culture of prepubertal goat COCs with denuded oocytes from large follicles secreting native OSF increased blastocyst yield, and the addition of a GDF9 antagonist did not hinder this effect.

In conclusion, oocytes from prepubertal goats respond positively to BMP15 regarding cumulus-oocyte communication, cumulus expansion and expression of EGFR in cumulus cells. This effect was not observed with GDF9.

Germinal vesicle configuration

In cattle, the oocytes that are usually used for IVEP, which come from 3 to 5 mm follicles, present their GV chromatin in various and unknown states of preparation for GV breakdown (reviewed by Sirard 2019). The GV undergoes various chromatin modifications and large-scale remodeling to regulate gene expression and cessation of transcription in preparation for meiotic resumption (reviewed by Tan *et al.* 2009). Thus, meiotic competence involves progressive chromatin condensation related to a decrease in transcriptional activity (Labrecque *et al.* 2015). In cattle, Sirard (2019) suggested that a specific GV chromatin configuration could be a good indicator of oocyte competence. Bovine oocytes were classified in four patterns of chromatin configuration from GV0 to GV3 which exhibit a progressive increase in chromatin compaction (Lodde *et al.* 2007), transcriptional silencing (Lodde *et al.* 2008), and global DNA methylation (Lodde *et al.* 2009).

Thus, bovine GV0 oocytes could not reach the MII stage, and GV1 oocytes reached the blastocyst stage at a lower rate (9%) than GV2 and GV3 oocytes (22% and 19%, respectively) (Lodde *et al.* 2007). In FSH-treated cattle, Sirard (2019) observed that with the coasting of all dominant follicles, the majority of them were at the GV2 stage at the time of collection and, accordingly, their blastocyst rate was close to 75%. In random ovaries recovered at the slaughterhouse, GV2 was found in 30% of oocytes (Dieci *et al.* 2016), which is similar to the blastocyst rates found in IVEP programs. In Sirard's (2019) model, the growing follicles would be in GV1, those in the plateau phase would be in GV2, and those in early atresia would be in GV3, featuring low, high, and medium blastocyst developmental competence, respectively.

Human GV oocytes are categorized as Class A, B and C based on increasing degrees of chromatin condensation. Class A oocytes (diffuse chromatin) do not reach the MII stage, Class B oocytes (intermediate configurations) predominantly reach the MII, and Class C oocytes (condensed chromatin) show no consistent correlation with meiotic resumption (Salimov *et al.* 2023).

Goat oocytes have been classified by Sui *et al.* (2005) in three stages of progressive GV chromatin condensation: GV1 (diffuse), GVn (net-like), and GVc (condensed). Only GV1 and GVn oocytes are transcriptionally active, whereas GVc oocytes are not (Sui *et al.* 2005). Cumulus-oocyte communication observed as transzonal projections (TZP) density increases with GV chromatin condensation up to the GVn stage, then it declines when the oocyte reaches the GVc stage (Ferrer-Roda *et al.* 2024*c*). In adult goats, oocytes from follicles larger than 3 mm display predominantly GVn configuration (Sui *et al.* 2005). Ferrer-Roda *et al.* (2024*a*) proposed that the GVc configuration tends towards atresia, while GVn leads towards ovulation. In prepubertal goats, oocytes from follicles smaller than 3 mm are evenly distributed among all GV configurations while follicles larger than 3 mm exhibit condensed chromatin (70.7% GVc) (Ferrer-Roda *et al.* 2024*a*). Considering the low blastocyst development of these prepubertal oocytes, we can suggest that most of the GVc oocytes are in the process of atresia, since they would never ovulate.

Sheep oocytes display three stages of progressive GV chromatin condensation: NSN (non surrounded nucleolus, diffuse), SN (surrounded nucleolus, intermediate stage of condensation), and SNE (surrounded nucleolus envelop, condensed). Russo *et al.* (2007) described a progressive chromatin condensation during follicle growth in adult sheep, with SNE oocytes being predominant in 3–6 mm follicles. In prepubertal sheep, most oocytes from follicles <3 mm displayed diffuse chromatin (63.7% in NSN), while most oocytes from follicles >3 mm exhibited highly condensed chromatin (59.2% in SNE) (Ferrer-Roda *et al.* 2024*a*). Cocero *et al.* (2019) found a similarly high level of GV chromatin condensation and global methylation in both prepubertal and adult sheep oocytes, all of which were recovered from follicles 3–6 mm in diameter.

Perhaps the most characteristic feature of GV in prepubertal large follicles is the highly condensed chromatin configuration, which may result from the atresia experienced by large follicles that do not have a path to ovulate. In any case, the intermediate stages of condensation that may be equivalent to the GV2 configuration in cattle (GVn for goats and SN for sheep) is lower than 20% in all analyzed small ruminant oocytes recovered from slaughterhouse ovaries (Ferrer-Roda *et al.* 2024*a*). Sirard (2022) indicates that follicles going into atresia may initially contain better competent oocytes but as GV chromatin compaction continues, developmental capacity decreases. If the chromatin is unable to synthesize new RNA, the lifespan of the oocyte is reduced.

Biphasic *in vitro* maturation (CAPA-IVM)

Oocytes with uncondensed chromatin are still in the process of accumulating transcripts indispensable for further development and have low developmental competence. IVM strategies with meiotic inhibitors such as CAPA-IVM provided extra time for GV chromatin condensation and improved oocyte competence. Capacitation-IVM, or CAPA-IVM, is a biphasic IVM system suggested by Gilchrist *et al.* (2016), designed to 'capacitate' the oocytes *in vitro*. CAPA-IVM consists of a pre-maturation (pre-IVM) phase with C-type natriuretic peptide (CNP) (which is the natural meiosis-inhibiting agent in the follicular fluid preventing meiotic resumption and cytoplasmic maturation) followed by a conventional IVM to induce maturation. CAPA-IVM has improved blastocyst development in mice (Romero *et al.* 2016), cattle (Franciosi *et al.* 2014), and goats (Soto-Heras *et al.* 2019*a*).

Oocytes from prepubertal females are generally collected from small-size antral follicles that have not yet completed the process of competence acquisition. Moreover, mechanically removing the COC from the follicle results in loss of the natural meiotic inhibiting environment, leading to spontaneous meiotic resumption via different molecular pathways (reviewed by Gilchrist and Thompson 2007) and closure of cumulus-oocyte communications (Luciano *et al.* 2011). In prepubertal goat oocytes, Velilla *et al.* (2005) observed that 50% of oocytes resume meiosis before IVM, during recovery from the follicle and preparation for nuclear assessment.

A pre-IVM with CNP maintains oocytes in meiotic arrest for 6 h in adult cattle (Zhang *et al.* 2017*a*; Soto-Heras *et al.* 2019*b*), sheep (Zhang *et al.* 2018; Varnosfaderani *et al.* 2020), and goat (Zhang *et al.* 2015; Assareh *et al.* 2022), leading to improvement in blastocyst development of these oocytes. Additionally, pre-IVM with CNP induced condensed chromatin configurations in cattle (Franciosi *et al.* 2014), mouse (Romero *et al.* 2016; Zhao *et al.* 2020), and goat (Soto-Heras *et al.* 2019*a*) oocytes. CAPA-IVM is currently in clinical use for IVF of women with polycystic ovarian syndrome (Vuong *et al.* 2020) and it increased transferable Day 3 embryos and good-quality blastocysts (Sánchez *et al.* 2017). However, some authors reported no effect of

CAPA-IVM on blastocyst yield (Franciosi *et al.* 2014; Santiquet *et al.* 2017; Soares *et al.* 2017; Assareh *et al.* 2022; Ferrer-Roda *et al.* 2024*b*). These differences in blastocyst development could be explained by the differences in the GV configuration at the time of pre-IVM. Thus, Dieci *et al.* (2016) demonstrated that pre-IVM improved the developmental capability of oocytes at early stages of differentiation (GV1) but was detrimental for oocytes at more advanced stages of development (GV2 and GV3).

CAPA-IVM with CNP improved the blastocyst yield of oocytes from small follicles but not from medium follicles in adult sheep (2–4 mm and 4–6 mm, respectively) (Varnosfaderani *et al.* 2020) and women (<6 mm and >6 mm) (Sanchez *et al.* 2019). In pigs, CAPA-IVM increased blastocyst development in medium follicles (3–6 mm) but it was detrimental in large follicles (6–8 mm) (Zhang *et al.* 2017*b*).

In oocytes from prepubertal goats, it is important to point out that the rapid meiotic resumption after oocyte liberation from the follicle necessitates the use of 500 μM IBMX in the slicing or aspiration medium to avoid meiotic resumption during oocyte recovery. In these oocytes, CAPA-IVM with 200 nM of CNP plus 10 nM of estradiol in the pre-IVM inhibited meiotic resumption for 6 h and induced condensed GV chromatin configurations while maintaining cumulus-oocyte communication and improving blastocyst development (3.2% and 17.2% for CAPA-IVM and control, respectively) (Soto-Heras *et al.* 2019*a*). CNP and estradiol can be combined for a synergistic effect, as estradiol enhances the meiotic-arresting activity of CNP by promoting the expression of its receptor in cumulus cells (Xi *et al.* 2018) and appears to be essential for long-term CNP-mediated meiotic arrest (Romero *et al.* 2016).

In conclusion, a CAPA-IVM protocol could significantly improve the oocyte competency of prepubertal females. However, the heterogeneity and the fragility of these variable GV oocytes made it difficult to predict their blastocyst output.

Reactive oxygen species and oxidative stress in the oocyte

Oxidative stress is the result of an imbalance between the production and elimination of the intracellular ROS and it is considered one of the main factors affecting the IVP embryos outcome. Oocytes also rely on a redox balance for their correct development. The process of oocyte maturation requires high energy supply in the form of ATP, especially for transcription, which in turn increases ROS generation by mitochondria (Chappel 2013). ROS are generated by cell metabolism, mainly by mitochondria due to electron leakage during oxidative phosphorylation, and continuously eliminated by the cell antioxidant defense mechanisms (reviewed by Hardeland 2005). A low physiological level is indispensable since ROS acts as second messengers in many cellular signaling cascades (reviewed by Dennery 2007). However, an imbalance in ROS production and elimination leads to oxidative stress (reviewed by du Plessis *et al.* 2008). ROS induces cell damage at different levels: lipid peroxidation which causes disruption of the cell membrane, protein damage like aggregation and degradation, and DNA damage such as deamination of nucleotide bases and strand breaks (reviewed by Evans *et al.* 2004). In the oocyte, oxidative stress affects oocyte nuclear maturation. Tamura *et al.* (2008) observed that inducing oxidative stress with H_2O_2 inhibits meiotic progression in mice oocytes. Oxidative stress causes aneuploidy, errors in the chromosome alignment and spindle morphology, and reduced ATP production due to mitochondria damage. Moreover, oxidative stress induces apoptotic cell death orchestrated by mitochondria and leads to oocyte ageing (Lord *et al.* 2013). Lastly, oxidative stress negatively affects fertilization by disrupting the membrane fluidity, which impairs the sperm-oolemma fusion (reviewed by Tarín 1996), and by interfering with calcium homeostasis which alters calcium oscillations after fertilization (Takahashi *et al.* 2003).

In the oocyte, enzymatic antioxidants present in the follicular fluid oxidize these ROS into harmless molecules (Ambekar *et al.* 2013). The follicular fluid contains non-enzymatic antioxidants, such as reduced glutathione (GSH), cysteamine, taurine, hypotaurine, vitamins (ascorbic acid, retinoic acid, tocopherol) (Guérin *et al.* 2001; Shi *et al.* 2009), melatonin (Shi *et al.* 2009) and Coenzyme Q10 (Turi *et al.* 2012). GSH, which works as a substrate for glutathione peroxidase (GPx), is considered the main non-enzymatic antioxidant in oocytes. Thus, high intraoocyte GSH levels are related to higher embryo development potential (de Matos *et al.* 2002).

Supplementation of IVM medium with antioxidants

In IVEP, oocyte ROS levels are higher than in *in vivo* conditions due to exposure to external deleterious factors and lack of follicle antioxidants (reviewed by du Plessis *et al.* 2008). Some of the external factors that promote ROS production *in vitro* are reviewed by Guérin *et al.* (2001) and du Plessis *et al.* (2008): oxygen concentration – in conventional IVM culture conditions it is 3–4 times higher than in the oviduct (Mastroianni and Jones 1965); metallic cations such as Fe^{2+} and Cu^{2+} – usually present in chemical products used for culture media (Nasr-Esfahani *et al.* 1990); visible light (Beehler *et al.* 1992); and amine oxidases – present in the sera used for culture media and released by dead spermatozoa (Shannon 1978; Parchment *et al.* 1990).

GSH is the main non-enzymatic defense system against ROS in oocytes (reviewed by Guérin *et al.* 2001) to neutralize H_2O_2. Oocytes from prepubertal females are particularly sensitive to ROS due to their low ability to synthesize GSH (Jiao *et al.* 2013). This study suggested that lower levels of GSH are responsible for the lower developmental competence compared to adults because it impairs male pronuclear formation, and resulting high ROS levels affect fertilization (Ca^{2+} homeostasis and cortical granules migration).

In our laboratory, we observed a lower GSH concentration in IVM oocytes from prepubertal goats, compared to ovulated oocytes from adults (5.6 vs 23.7 pmol/oocyte) (Rodríguez-González *et al.* 2003). Because of the high percentage of abnormal zygotes after IVF-prepubertal oocytes, we have tested the use of thiol compounds for IVM, including GSH and its precursors (cystine, cysteamine, N-acetylcysteine, and β-mercaptoethanol), with the conclusion that cysteamine increases the GSH intracytoplasmic concentration compared to immature oocytes and IVM-oocytes without antioxidants (7.7, 4.2 and 5.6 pmol/oocytes, respectively), leading to improved blastocyst yield and quality (Rodríguez-González *et al.* 2003). Nowadays, cysteamine is probably the most commonly added antioxidant to IVM medium with a dose of 100 μM. Cysteamine has proved to increase GSH content and blastocyst rate of sheep (de Matos *et al.* 2002) and cow oocytes (de Matos *et al.* 1995).

Several studies have been testing melatonin as an antioxidant for IVEP programs due to its more powerful action than other IVM supplements. In prepubertal goats, oocytes matured with melatonin develop to the blastocyst stage at a higher rate than a control IVM without antioxidants (28.9% and 11.7%, respectively) (Soto-Heras *et al.* 2018). Moreover, we determined that melatonin not only reduces the intracytoplasmic ROS level in prepubertal goat oocytes but also improves oocyte mitochondrial activity and ATP content (Soto-Heras *et al.* 2019*c*).

Different biological active components from plant and algae of known antioxidant power have been tested in oocyte IVM, reporting an improved oocyte developmental competence by oxidative stress reduction (reviewed by Soto-Heras and Paramio 2020).

In prepubertal goats, the supplementation of IVM media with resveratrol (3,4,5-trihydroxy-*trans*-stilbene), a small polyphenol synthesized by several plants such as nuts, mulberry and grapes, and crocetin ($C_{20}H_{24}O_4$), an active constituent of saffron (*Crocus sativus* L.) which as other antioxidants protects cells from the damaging effects of ROS, has been studied. Resveratrol has shown a significant increase in blastocyst production in BCB+ oocytes compared with no-resveratrol medium (20.1% and 6.8%, respectively) but not in BCB- oocytes (Piras *et al.* 2019). The supplementation of crocetin during IVM has not improved blastocyst development obtained by both ICSI and parthenogenic activation, despite the significant reduction in intracytoplasmic ROS levels (Menéndez-Blanco *et al.* 2020*a*).

Our conclusion is that some antioxidants have shown a ROS neutralizing power and an overall improvement of oocyte developmental competence for JIVET programs. However, the results in terms of blastocyst yield are still variable among studies and experiments. Each laboratory should choose the best antioxidant and concentration based on their specific environmental conditions, technical resources and origin of the oocytes.

Conclusions

In conclusion, the ovaries of small ruminant prepubertal females contain a high number of antral follicles, most of

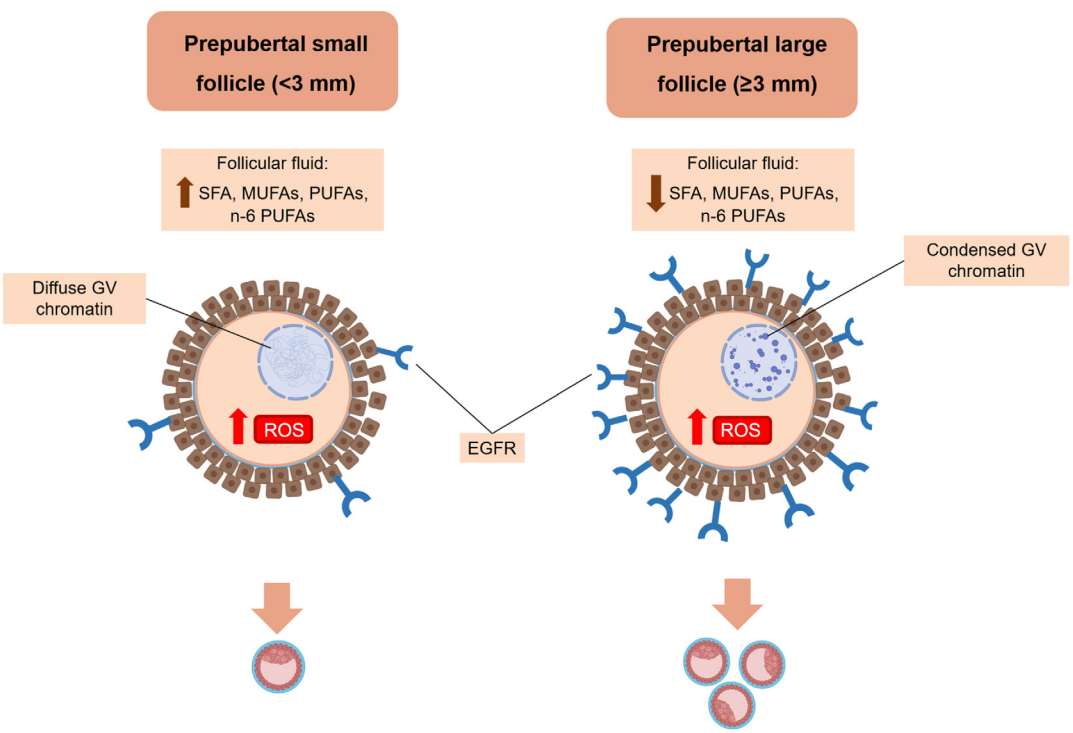

Fig. 3. Markers of oocyte competence according to the follicle sizes of prepubertal goat oocytes.

which are smaller than 3 mm and their oocytes after fertilization exhibit a high variability and low blastocyst rates across experiments. Selecting oocytes based on their diameter or using BCB staining significantly enhances blastocyst production. A clear relationship exists between follicle size, oocyte diameter, and developmental competence, as previously observed in adult females.

In prepubertal goat and sheep, oocytes retrieved from follicles larger than 3 mm are capable of developing into blastocysts at rates comparable to those from adult females. These larger follicles also exhibit higher expression of EGFR in cumulus cells and a greater proportion of GV oocytes with condensed chromatin, both of which are considered reliable biomarkers of oocyte competence. Perhaps the most characteristic feature of GV in prepubertal large follicles is the highly condensed chromatin configuration, which may result from the atresia experienced by large follicles that do not have a path to ovulate. Fig. 3 represents the markers of oocyte competence according to the follicle sizes of prepubertal goat oocytes.

To improve the developmental potential of prepubertal goat oocytes, a biphasic *in vitro* maturation (CAPA-IVM) protocol has been tested. This approach significantly increased EGFR expression in cumulus cells, the proportion of GV oocytes with condensed chromatin, and blastocyst formation rates. Furthermore, supplementation of the IVM medium with oocyte-secreted factors was evaluated. BMP15 enhanced EGFR expression and improved cumulus-oocyte communication, as indicated by increased transzonal projection density, whereas GDF9 showed no beneficial effect on oocytes from small ruminants.

Due to their fragility and susceptibility to oxidative stress, the inclusion of antioxidants in the IVM medium is essential for maintaining the viability of these oocytes.

Although this study focuses on small ruminants, the findings regarding oocytes from small follicles may have broader implications and could be applicable to other species, including humans, potentially advancing reproductive technologies across a range of species.

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