In vitro culture of sheep early-antral follicles: Milestones, challenges and future perspectives

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In vitro culture of sheep early-antral follicles: milestones, challenges and future perspectives

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Abstract

Early antral follicles (EAFs) can represent an easily exploitable reserve for the production of mature oocytes in livestock, as they are more abundant than antral follicles (AFs) and need less culture period compared to the other pre-antral follicles (PFs).

Despite some impressive achievements, maturation, cleavage, and blastocyst rates are still far below the standard of in vitro embryo production system (IVP) in ruminant species. The difficulty is related to the development of suitable in vitro culture systems tailored with nutrients, growth factors, and other signaling molecules which should supported oocyte growth. In this review we focus on in vitro development of sheep EAFs to provide an informative reference about current research progress in the in vitro culture systems, medium supplementation, challenges, and future perspectives.

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1. Introduction

In sheep, throughout the reproductive life span around 80,000 primordial follicles are recruited for growth, and only a small number of this genetic pool will ovulate, while the rest of them undergo atresia during folliculogenesis [1]. This represents an untapped potential that can be used to preserve the fertility of high genetic merit animals and endangered species [2]. However, the exploitation of the entire ovarian germinal cell reservoir is not yet possible. In vitro growth of primordial follicles in the ovarian cortex to produce mature oocytes is still at an experimental stage [3]. Oocytes at early stages of their differentiation enclosed in preantral and small antral follicles are discarded from routine in vitro embryo production (IVEP) protocols as they must complete growth and development to attain full meiotic and embryonic developmental competence. The population of early antral follicles (EAFs), instead, may represent a reserve of oocytes close to completing the growth phase, which might be more easily exploited in vitro and could increase the number of female gametes dedicated to IVEP [4]. Being larger and at more advanced stage of development, EAFs require a shorter culture period to achieve the developmental competence than secondary follicles (≤300µm vs 350-500µm, Fig 1).

Accordingly, several efforts have been made to develop a suitable in vitro culture system that can provide an optimal environment for EAFs to survive and develop. For instance, after in vitro culture of EAFs variable oocyte maturation rates have been reported in cow (5–79%) [5, 6, 7,], goat (24.1-46%) [9] and pig (58-68%) [10, 11]. Although these results are promising, only a limited number of embryo have been produced in cow and goat [7,12] with no live born.

In sheep, after developing the follicle isolation methods [13,14], the first obtention of matured oocyte (MII) from in vitro culture of late secondary and EAFs (follicle sizes: 150–250µm and 250-400µm) was reported by Tamilmani et al (2005). Subsequently, successful of in vitro oocyte maturation increased up to 68% [16]. However, despite recent promising reports [17,18], no successful embryo development beyond the morula stage (16.28% of cleaved cells) has been reported until now [16]. In general terms, in this species, the overall efficiency is still low and only a variable number of matured oocytes and embryos with no successful parturition have been reported. The success of this procedure depends on several factors including: initial follicle/oocyte size [16], medium supplementation [19], reproductive state of the ovary donor [20], culture period, frequency

of medium replacement [21], the composition of culture medium [16], etc. The low and variable outcomes indicate that the culture conditions still need to be optimized and standardized [22,23].

In this review, we briefly resume the physiological aspect of oocyte growth and follicle formation. Then, we focus on the factors involved in the in vitro culture of EAFs with emphasis on medium supplementations to provide a new perspective for identifying the base culture medium, and supplements (hormones, growth factors, etc). To do this, we reviewed the published papers on sheep as an animal model which is cost-effective, more accessible, and has comparable characteristics to human ovaries [24].

2. Basic aspects of folliculogenesis in sheep

In sheep, folliculogenesis initiates with the breakdown of germ cell clusters and the formation of primordial follicles at approximately 65-75 days of gestation [25]. The primordial follicle contains an oocyte surrounded by a single layer of squamous pre-granulosa cells which is in turn surrounded by a basal membrane; this forms the smallest ovarian follicle units and it is defined as the ovarian reserve pool [26]. These follicles continuously grow and leave the reserve pool of quiescent follicles (recruitment or primordial follicular activation) to become transitional and primary follicles (growing phase). Subsequently, they develop to the secondary follicles by the addition of a second layer of granulosa cells, the initial deposition of zona pellucida (ZP) and the beginning of theca cell layer formation [27,28].

As the follicle diameter and hence the distance between the oocyte and blood vessels increases, the antrum filled with follicular fluid is formed among the granulosa cells to convey nutrients, gases, and signaling molecules to the innermost cells and oocyte [29,30]. At this stage, the tertiary follicles (also knowns as early antral follicles) are formed and the oocyte diameter reaches around 110μm (Fig 1) [31, 32]. Also at this stage, the granulosa cells are gonadotrophin responsive and in proliferation while embracing a meiotically arrested oocyte [20,30]. During this process, the oocytes are still growing, meiotically non-competent, transcriptionally active, display low levels of global DNA methylation and store a large amount of RNA [2,33,34]. Additionally, a bidirectional communication between cumulus cells and oocytes takes place, as well as changes in carbohydrate and lipid metabolism.

Throughout the secondary/EAF transition the chromatin configuration changes from diffuse chromatin (non-surrounding nucleus-NSN pattern, Fig2) to the condensed chromatin configuration (surrounding nucleolus-SN pattern, Fig 2). In the more advanced phase of oocyte development (124.7 ± 6.5µm), they display a new pattern of chromatin configuration in which the condensed chromatin appear partly around the nucleolus and partly close to the nuclear envelope (SNE configuration, Fig 2) [35,36]. Also, a phase of structural changes and spatial differentiation occurs as the follicle grows including Golgi, endoplasmic reticulum and mitochondria position [32]. These changes require the appropriate expression of numerous genes such BMP15, DNMT1, GDF9, MOS, ZAR1 and orchestrated communication between the two main compartments (oocytes and granulosa cells) [37,38].

Regulation of this transition includes a complex interaction among endocrine, paracrine and autocrine factors which in turn affects steroidogenesis, angiogenesis, follicular atresia, oocyte growth, and maturation as well as the proliferation and differentiation of follicular cells.

As the process of maturation progresses, one or several follicles (in mono- or poly-ovulatory species, respectively) are selected and undergo final maturation. In sheep, it takes 154 to 165 days for the primordial follicle to develop into the early antral follicle stage (0.5 mm in diameter- Fig 1), and then an additional five days to reach the small antral follicle stage (2.2 mm in diameter) [39, 40]

3. Isolation of EAFs (methods and recovery rate)

Among the different methods used for follicle isolation (mechanical, enzymatic, or combination of both), microdissection is the most common for EAFs isolation. In this method, follicles are isolated from a thin ovarian slice (2-3 mm) by using two gauge needles (22-26 G) fitted to 1mL syringe barrels [31,41]. In comparison with other methods, microdissection is simple, cheap and can maintain the integrity of the follicle, preserving interactions between oocytes/granulosa cells and minimizing the risk of rupturing the basal membrane [42,43]. However, due to the dense connective tissue surrounding the EAFs, the isolation procedure is difficult and time-consuming [44]. Accordingly, the number of isolated EAFs is limited. In contrast, the enzymatic method can easily isolate more follicles by incubation of ovarian cortical fragments with adequate concentration of specific enzymes (hyaluronidase, collagenase or trypsin) [45–47]. This method, besides being more

expensive, may cause damage to the follicles and oocytes, especially in the basement membrane [48,49]. Therefore, the mechanical method is more common for the isolation of ruminant EAFs.

The number of isolated follicles by mechanical methods may be affected by many factors such as operators, breeds, species, and seasons [34,39,50,51]. For example, in seasonal breeders, such as the sheep, there are more PFs and fewer AFs during the anoestrous period which suggests that the total number of growing PFs is increasing in anoestrus and falling in the breeding season whilst the antral follicle population follows an inverse fluctuation [39]. Accordingly, the number of sheep EAFs in the reproductive season can vary from 30 follicles in high ovulation breeds to 8-12 follicles in low ovulation breeds [39]. Despite the high number of EAFs in the ovaries, the recovery rate by using the usual mechanical methods is still low and labored [52]. In this regard, Chakravarthi et al (2015) indicated that 5 to 10 preantral, 5 to 10 early antral, 3 to 5 antral, and 1 to 2 large antral follicles could be routinely isolated from each ovary. However, the breed and season are not mentioned. In addition, age, hormone concentration, ovarian size and presence or absence of corpus luteum influence the quantity and quality of recovered follicles from slaughtered animals [51,54]. For example, during the breeding season, a big part of the ovaries is occupied with the corpus luteum which limits the accessibility to the ovarian cortex. Therefore, due to the abovementioned variations and also to the isolation methods, the recovery rate of late secondary follicles and EAFs from each ovary can vary from 4-6 to 5-10 follicles, respectively [53,55].

4. In vitro culture of sheep EAFs

4.1 In vitro culture systems of EAFs

Selecting the appropriate in vitro follicle culture system based on the follicle stages (primary, secondary and etc.) and the desired culture duration is crucial for ensuring optimal growth [56,57].

For the EAFs, in vitro culture of isolated follicles is the most established technique, in which the whole follicles (with the intact basement membrane) are cultured either in a two-dimensional (2D) or a three-dimensional (3D) system [42,56].

The 2D follicle culture systems have great diversity, such as the droplet method, microplate culture, and culture plate inversion culture [58,59]. Among those, the microplate method is the most commonly used due to its versatility and ease of use [56,60]. However, this system showed

to be inefficient in maintaining the normal architecture of follicles especially during the long-term in vitro culture (more than 4 days) [56]. This is due to the GCs adhering to the bottom of the culture dish, which can cause loss of the original spherical shape and disrupt gap junctional communication [61,62]. Despite this, the 2D culture system is the only ones used in published literature for in vitro culture of sheep EAFs (Table 1).

On the other hand, the 3D culture system has gained attention as an alternative, especially for long in vitro culture of follicle, as follicles are embedded in an extracellular matrix (alginate, hyaluronan hydrogel, PVP, and etc.) that provides the right balance between rigidity and elasticity to support the spherical shape of the follicles and prevent disruption of gap junctions [19,60,62–64]. However, the use of 3D culture system for in vitro culture of large follicles such as sheep EAFs (which are 350-500µm in size) faces certain challenges. One of the main issues is the presence of the follicle basement membrane and limited penetration of oxygen and nutrients (less than 200µm) in the culture medium which may restrict their diffusion to the central part of the EAFs [65,66]. As a results, a new approach has been developed that involves using isolated cumulus oocyte complexes (COCs) to improve the exchange of gases and nutrients between the medium and the oocyte [67,68]. Positive outcomes from this culture system have been obtained in cattle, with embryo production rates reaching 30% of cleaved embryos [69], there is currently insufficient evidence to support its effectiveness in sheep.

4.2 Culture conditions

In vitro culture conditions are critical for the growth and survival of follicles in a laboratory setting. These conditions include the duration of the in vitro culture, concentration of gases, volume of culture medium, replacement interval of culture medium, type of culturing dish and medium composition.

4.2.1 Duration of in vitro culture

The time required for a follicle to reach the optimum size of antral follicle, in which the oocyte inside is able to resume meiosis, is species-specific and can vary depending on the follicular phase, culture medium, and method employed [68]. In sheep, it takes approximately five days for EAFs containing immature oocytes (110 ± 5µm) to achieve full meiotic competence through in vivo

development (130 ± 5µm- Fig 1) [59,70]. Nevertheless, different durations of EAFs culture were reported in the published literature to achieve full meiotic competency (Table 1). This variability could be attributed to differences in the size of EAFs used (ranging from 250 to 500µm), season, breed or cultured conditions.

4.2.2 Concentration of gases

The composition and concentration of gases, especially oxygen, are critical factors that must be adjusted to mimic in vivo conditions. Low oxygen levels can lead to oocyte hypoxia, especially when there are multiple layers of granulosa cells around the oocyte, which can impede oxygen distribution. Conversely, high oxygen levels can trigger oxidative stress and apoptosis [71,72]. Studies on human follicular fluid have shown that the mean dissolved oxygen levels are low at the initial formation of the early antral follicle (~1.5 vol%) and peak in the later phase (~6.7 vol%) as the follicle grows [73]. However, in all studies on in vitro culture of sheep EAFs (Table 1) and even other animals like cow [67] and goat [12], a higher concentration of oxygen (5% CO_2 in air or 18.6% oxygen) was used than the reported level of dissolved oxygen in follicular fluid [73,74]. This apparent paradox may be a consequence of oxygen's slower diffusion through aqueous media and the underappreciated effects of cell density, media volume, and barometric pressure on pericellular oxygen concentration in cell culture systems [74]. Therefore, since no studies have determined the specific concentration of oxygen required for in vitro culture of EAFs, it would be of interest for such experiments to be conducted in order to more accurately mimic in vivo conditions in the culturing system.

4.2.3 Volume of culture medium

The volume of the culture medium should be carefully adjusted to meet the nutrient demands of the cells, prevent osmolarity fluctuations, absorb toxic substances produced by the cells, and maintain other beneficial factors secreted by follicle cells [75]. In previous studies on in vitro culture of sheep EAFs, researchers typically used a microdrop culture system, in which small volumes of medium (10-100µl- Table 1) were covered with mineral oil to prevent osmolarity fluctuations. However,

mineral oil poses a high risk as it can reduce bioavailability of liposoluble substances (e.g. progesterone and estradiol), and release several toxic substances (Triton X, alkenes, and aldehydes) into the medium [76]. To avoid these undesirable effects, an alternative oil-free culture system has been developed in other species, where a high volume of medium (200 µl) is used to prevent osmolarity changes [67,77]. However, using a high volume of medium can dilute specific compounds derived from follicular cells and can affect gas exchanges due to the high volume and depth over the follicle [74].

Generally, determining the optimal volume of in vitro culture medium is challenging and several variation such as the type of culture dish, gas pressure and liquid surface area should be considered [74,78,79]. Therefore, since the medium is an essential part of cell culture, further studies are necessary to determine the best volume of medium for in vitro culture of EAFs.

4.2.4 Replacement interval of culture medium

Replacing the culture medium after specific time periods is necessary to avoid nutrient exhaustion and the accumulation of toxic products. The optimal replacement interval of the culture medium varies depending on several factors, such as the type of medium used (TCM or αMEM), the culture system (2D or 3D), and the specific requirements of the EAFs [21,75]. In general, it is recommended to replace half of the medium every 2 days for most in vitro culture systems, including microdrop and oil-free culture systems [31,55]. Other authors used a longer replacement intervals (3 or 6 days) to minimize fluctuations in the culture conditions [15,21]. While medium replacement may result in better growth and development, it could also remove benefit substances from the medium. Periodic addition of fresh medium without removing the old medium can thus be an alternative option [75].

4.2.5 Type of culture dishes

To the best of our information, most researchers on the in vitro culture of sheep EAFs has utilized a petri dish with diameters of 35 and 60 cm (Table 1). Few authors used 96-well microplates with either V-shaped [59,80,81] or flat bottom [67]. However, we did not find any additional information comparing the impact of petri dish size, material, or type on EAF culture outcomes.

Nevertheless, it may be beneficial to consider some general characteristics of culture dishes to optimize successful EAF culture.

Among cell culture dishes, polystyrene dishes have become popular due to their ease of use and low cost. However, their hydrophobic surface can make it difficult for cells to attach [82]. However, long in vitro culture of follicles and COCs revealed that granulosa cells can attach to the petri dish bottom, leading to gap junction dysfunction which plays an essential role in oocyte growth and development. Due to this, various systems including encapsulation of follicles [83,84], use of treated/hydrophobic membranes [16,85,86], or daily transfer of the follicles to a new well [87] have been used to prevent attachment and preserve the structural integrity of follicles. The efficiency of these methods still need to be evaluated.

To choose the best type of culture dish, it should be noted that the petri dish requires a high ratio of mineral oil to medium, which can sink the follicles in a pool of toxic substances and absorb liposoluble substances from the medium [88]. Alternatively, in the case of 96-well microplates, osmolarity can be regulated without mineral oil by adding water to the corner of the plate. However, it is important to take into account that the "edge effect" (where evaporation from the corner and edge wells is greater than that from the interior wells) could affect the usability of these plates [89]. Future studies will likely provide more information for selecting the best culture dish.

4.3 Medium composition

Successful in vitro culture of EAFs requires the provision of nutrients, cytokines, growth factors, and hormones in a tightly regulated environment that mimics the physiological conditions of follicular development. Several commercial media have been investigated for the in vitro culture of EAFs, including α-MEM, TCM-199, and Waymouth (Tables 1). Among these, TCM-199 has been found to better support the development of sheep EAFs compared to the other media [90,91]. TCM-199 contains a richer composition of nutrients and growth factors that promote cell viability and follicular development [92,93]. As an alternative to TCM-199, α-MEM can be used if fresh medium is continuously added. Indeed, to identify the best in vitro culture medium for sheep EAFs, the supplementations and replacement interval of the medium should be carefully considered [55].

In addition to the choice of the medium, the supplementation of antioxidants, growth factors and developmental stage-dependent hormones is critical for the successful in vitro culture of EAFs

[21,34,42,94]. The successful in vitro culture of EAFs is indeed a complex process that requires a carefully balanced medium composition and tight regulation of the culture conditions. However, the high cost of these components may limit their use in many laboratories.

4.3.1. Hormones and growth factors

Folliculogenesis is a complex phenomenon in which several intra and extra ovarian factors act to maintain the development of follicles. The concentration, corresponding receptors, and balance between these factors are stage-specific and vary between follicular compartments and species [2,16,95]. Therefore, exploring the follicular requirements and supplementing the in vitro culture medium with those substances could improve in vitro growth of sheep PFs. The following sections will describe only the hormones and growth factors that have been used in the in vitro culture of late secondary follicles or EAFs in sheep.

4.3.2. Gonadotrophin hormone (FSH and LH)

PFs development in sheep is largely independent on gonadotropin stimulation [15]. The presence of FSH receptors in the PFs may explain some biological roles of this hormone in folliculogenesis [96]. FSH stimulates granulosa cell growth and proliferation, induces antrum formation [97], improves oocyte growth, survival of secondary follicles, mitochondrial activity, oocyte maturation [16] and stimulate intense DNA synthesis [47]. Also, this hormone interacts with other hormones (such as IGF-1) to support granulosa cell differentiation and function [98].

In sheep, researchers have used different concentrations of FSH to support in vitro development of late secondary and EAFs (Table 1). However, Tamilmani et al., (2005) reported that FSH concentration above 2 µg/ml is toxic for sheep PFs. This difference could be due either to the variation in size of PFs, source of hormone (recombinant or purified form), the origin of the FSH (human, ovine, bovine, rat, or porcine), and other factors in the culture medium [13]. Conversely, luteinizing hormone (LH) as the other member of gonadotropin hormones does not have receptors until the preantral stages (15) and has a negative effect on in vitro production of sheep PFs [15,99]. However, recently Reddy et al., (2021), indicated that supplementation of LH during the first two days (0-2 days) of culture increased follicle diameter, antrum formation, and

IVM rate. These controversial reports may indicate that probably normal follicle development needs stage-specific hormone supplementation.

4.3.3. Growth hormone (GH)

GH is produced and secreted by the pituitary, gonads, uterus, placenta, and mammary glands and binds to its receptors in ovarian granulosa, theca, oocytes, and cumulus cells [100]. In sheep, it has been shown that supplementation of GH to culture media, independently or in combination with the other substances (IGF-I, T4, and FSH) can influence optimal follicular development, survival, increase antrum formation, meiosis resumption, and oocytes maturation [16,21]. Furthermore, GH can improve mitochondria activity and subsequently the quality of oocytes and fertilization rate [101]. Due to this, several researchers suggested 1mIU/ml of GH (Table 1) as the best concentration to support in vitro development of sheep EAFs [16,90,102,103].

4.3.4. Thyroxin (T4)

It is widely acknowledged that T4 has a significant impact on ovarian folliculogenesis [104]. Studies conducted on sheep have demonstrated that T4 can enhance in vitro development, meiotic competence, survival, and ultrastructural integrity of PFs [90,99,105]. Notably, the magnitude of these effects is size-dependent, with larger PFs exhibiting a more substantial response to T4 [16]. Based on this, it is reasonable to assume that T4 would also have comparable effects on EAFs (350-500µm).

Previous research by Arunakumari et al. (2007) has shown that T4 can support the maturation of oocytes from cultured large PFs (250-400µm) to the MII stage, although only in a small proportion of cases (Table 1). Furthermore, when T4 is combined with FSH, it supports better in vitro growth, antrum development, and subsequent maturation of oocytes to the MII stage in these PFs.

Given the synergistic effects of T4, FSH, IGF-I, and GH on the growth rate, antrum formation, and follicle diameter [99], it is worth considering the potential of T4 for promoting the in vitro development of sheep EAFs.

4.3.5. Estradiol-17β

Estradiol has specific intracellular receptors which are expressed in the granulosa cells [106]. The limited studies on in vitro culture of EAFs in cows and pigs showed some positive effects of this hormone on meiotic and developmental competence of oocytes, cumulus-expansion, and cumulus-oocyte integrity [11,107]. There is no report on the effects of this hormone on in vitro culture of sheep EAFs. However, recent studies on the late secondary follicles suggested that estradiol promotes follicle growth [90], antrum formation [17], nuclear maturation [90], and inhibits follicle atresia by reducing the oxidative stress [17,108].

In this context, Reddy et al., (2021) indicated that the supplementation of LH and estradiol (5 ng/ml- Table 1) during the first two days of follicle culture supported a better average increase in diameter and antrum formation. Furthermore, the isolated oocytes at the end of the culture showed a higher in vitro maturation rate.

4.3.6. Leptin

Leptin regulates nutritional status and energy sufficiency in all stages of the ovarian follicle by binding to its receptors in the oocytes and cumulus cells [109,110]. Moreover, it participates in regulation of ovarian folliculogenesis indirectly via control of luteinizing hormone and FSH secretion [111].

It has been suggested that supplementation of medium with the physiological concentration of leptin in plasma (0.5-2 ng/mL) could be sufficient to support the in vitro development of sheep EAFs, while the higher concentrations (10ng/ml and 25ng/ml for 300-450µm and 240–270µm, respectively- Table 1) were recommended for the smaller follicles [102,109,110]. In sheep leptin supplementation to EAFs culture medium has been shown to promote follicular development, stimulate its receptor expression [102,109], improve meiotic resumption, follicular survival, antrum formation, oocyte growth, attenuating mitochondrial dysfunction and oxidative stress [31,110].

4.3.7. Melatonin

Sheep follicular fluid contains melatonin derived from blood and self-secretion which increases with follicle size [112]. It has been reported that supplementation of 500-1000 pg/ml melatonin in the culture medium of sheep EAFs stimulates antral cavity formation, enhances the development of secondary and early antral follicles, increases mitochondrial activity and percentage of fully grown

oocytes [18]. Moreover, it acts not only as a hormone but also as an efficient antioxidant that can enhance the development and decreases ROS levels of oocytes from in vitro-grown EAFs [18,113]

4.3.8. Insulin-like growth factor-I (IGF- I)

IGF-I plays an essential role in the proliferation and differentiation of granulosa cells and ovarian response to FSH action [114,115]. In sheep, it has been detected in the oocytes and granulosa cells at all follicular stages and acts as a stimulatory growth factor [116]. It can increase follicular diameter, oocyte maturation rates, antrum formation, cell proliferation, and reduce follicular atresia and DNA fragmentation [16,116,117]. It also has a synergic effect with GH, T4, and FSH on follicle and oocyte development in sheep [16,116]. Therefore, IGF-1 appears to be among the most important growth, differentiation and survival factors which can protect granulosa cells from apoptosis and supports cell differentiation and function [98].

4.3.9. Transforming growth factors (TGF-α and TGF-β)

The TGF family has two main members (TGF-β and TGF-α) with a wide range of biological activities related to cell proliferation and differentiation depending on the cell type and growth factor environment [118]. In sheep, TGF-β has been reported to be produced in the ovarian theca cells at all follicular stages and affects luteinizing granulosa cells [119]. However, its effects on the in vitro development of late secondary follicles (250-400µm) and oocyte development are inconsistent [16].. These results may be related to the presence of other components and growth factors in the medium, as the inhibitory effect of the TGF-β can be attenuated by the simultaneous presence of IGF-I [16].

TGF-α, another member of the transforming growth factor was reported to induce (2.5ng/ml) in vitro growth of late secondary follicles (250-400µm), new DNA synthesis, increase follicle size, antrum formation, and meiotic maturation [15,47].

4.3.10. Growth differentiation factor- 9 (GDF-9)

In the sheep ovary, GDF-9 has been found to be expressed in all follicular categories indicating its direct involvement in folliculogenesis, oocyte growth and meiotic resumption [38,120,121]. However, the effects on follicular survival and apoptosis are controversial [116,122].

4.3.11. Fibroblast growth factor 2 (FGF-2)

FGF-2 is a member of the fibroblast growth factors family [123]. In sheep, it is produced in PFs and granulosa cells of secondary and antral follicles [124]. Therefore, it may have an important role in the regulation of PFs development including DNA synthesis, antrum formation, and nuclear maturation of the oocytes in the cultured follicles [125,126]. Also, it has positive effects on cell proliferation, in vitro growth of PFs, and decrease apoptosis [124,127]. It has been indicated that 10 and 50 ng/ml of FGF-2 as the minimum and toxic concentrations for in vitro culture of sheep PFs, respectively [90]. However, further research needs to be done to indicate the optimum dose for improving nuclear maturation in the sheep PFs.

4.3.12. Epidermal growth factor (EGF)

Immunostaining studies of sheep oocytes and granulosa cells confirmed the presence of EGF protein in all follicular stages [124]. However, during the transition from secondary to antral follicle stage a reduction in the EGF production by the granulosa cells was reported [124]. The EGF is involved in the regulation of several ovarian processes and the maintenance of normal ultrastructural characteristics of sheep follicles [91].

Previous research in sheep revealed that adding EGF to the culture medium can improve in vitro survival [128], stimulate follicle growth, induce DNA synthesis [47] and an increase in oocyte diameter [91]. Despite of these benefits, the oocytes cultured with EGF (50ng/ml) are unable to reach the MII stage [15,124]. These results suggest that EGF might initiate the growth of sheep PFs, but requires other factors to sustain or complete the process [15,47].

4.3.13. Leukemia inhibitory factor (LIF)

The LIF is an important cytokine in sheep folliculogenesis which can affect cell survival, proliferation, or differentiation depending on the cellular context [129,130]. It has been shown that the levels of LIF in the follicular fluid increase progressively during follicular development and

influence oocyte growth, maintenance of follicular viability [131], and modulated the differentiation of granulosa cells [130]. Also, when associated with FSH, LIF increases antrum formation [131], expression of genes involved in the oocyte-granulosa dialogue, modulates the effects of culture on follicle development, and improves oocyte meiotic competence [130]. On contrary, Cadoret et al (2021) did not show a significant change in follicle growth, antrum formation, and follicle survival in sheep. Also, compared to the previous report, they showed a higher maturation rate for oocytes derived from in vitro culture of PFs (200µm<) in the presence of 50 ng/mL LIF (56% vs 29%) [131]. The discrepancy may be due to differences between the culture systems, including the origin of FSH or LIF [130].

5. Antioxidant

Sheep oocytes contain a high level of lipid microdroplets (about 89 ng) including saturated fatty acids (45–55% of total fatty acids in oocytes) and monounsaturated fatty acids (MUFA: 27–34%) or PUFA (11–21%) [132,133].

The combination of this characteristic and physical stress during in vitro culture makes sheep oocytes susceptible to oxidative stress and interrupts the balance between pro- and anti-apoptotic factors [134,135]. Consequently, they can negatively affect meiotic spindle formation, oocyte maturation [136,137], and follicle development [12].

Due to this, a great variety of antioxidants such as ascorbic acid, transferrin, selenium, Gallic acid [55], coenzyme Q10 [138], rutin [139], kaempferol [140], protocatechuic acid [141] melatonin and insulin [18] have been tested to diminish the negative effects of oxidative stress in sheep follicle culture (Table 1). Regardless of whether they are used alone or in combination with other antioxidants, they improved follicular development by promoting granulosa cell viability, maintaining follicle morphology, and inhibiting apoptosis [139]. Altogether, adding the antioxidants at an appropriate concentration/combination is needed to reduce oxidative stress during long-term in vitro culture of follicles [34].

6. Challenges and prospective

In vitro culture of EAFs is a promising approach for fertility preservation and assisted reproductive technologies. However, several challenges must be addressed before this technique can be applied. One of the main challenges is related to the limited number of retrievable follicles (5-10/ovary). The population of EAFs can be affected by various factors, such as donor age, nutrition, season, and reproductive stage, making it difficult to predict the number of retrievable EAFs within each ovary. The current methods for isolating EAFs are also labor-intensive and have a low recovery rate [52]. In addition, since oocyte diameter is not directly proportional to follicular size [142], the variation in the reported size of EAFs may also affect the in vitro culture outputs (Table 1). Therefore, the proper identification of the developmental stage of isolated EAFs, and its relation to oocyte maturation stage, will greatly enhance current fertility preservation strategies.

Furthermore, providing adequate nutrients and creating an optimal environment for EAFs is challenging. Sheep EAFs are relatively large (350-500μm), which may restrict the diffusion of oxygen and nutrients and limit their ability to reach the follicle core [65]. The in vitro culture system must also provide sufficient support to retain the spherical shape of the follicles and supply various nutrients, antioxidants, hormones, and growth factors in the best concentration, combination and timing [13,39,40]. Additionally, the effect of the in vitro culture medium and environment on the epigenetic changes should be considered since the oocytes acquire their epigenome during their growth [34,59].

Despite these challenges, exploring novel isolation methods combined with bioengineering technology, such as microfluidic technology [143], and supplementation of medium with predefined substances (e.g. antioxidants), signaling molecules (e.g. exosomes and hormones), and co-culturing with the somatic cells (cumulus or granulosa cells) hold immense promise for advancing this field and improving outcomes [41,60,144,145].

7. Conclusion

In vitro culture of EAFs will probably become a key strategy for the preservation of fertility in mammals in the future. Accordingly, it is being developed as a new method for increasing the availability of competent oocytes. However, designing a suitable culture system is complicated as several parameters should be consider to preserve oocyte quality while supporting the growth and architecture. For example, several factors that contribute to follicular development are still unclear, such as the type of culture medium, hormones, and growth factors to be used and the ratio of each.

In this regard, other factors such as season, nutrition, age, and follicular requirements which seem to be stage-specific and species-specific should be considered. Overall, the results are promising but more research is needed to develop or introduce a new follicle isolation method, in vitro culture systems (2D or 3D, single or co-culture) and other signaling factors.

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Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Mohammadreza Ebrahimi: search strategy, studies reviewed, writing – original draft, wrote the manuscript. **Maria Dattena:** writing- reviewing and editing, and approved the final version of the manuscript. **Alberto Maria Luciano:** designed the study, reviewed studies, and contributed to the original draft writing. **Sara Succu:** writing- reviewing and editing. **Sergio Domenico Gadau:** writing- reviewing and editing. **Laura Mara:** writing- reviewing and editing. **Fabrizio Chessa:** writing- reviewing and editing. **Fiammetta Berlinguer:** designed the study, conducted the search strategy, reviewed studies, contributed to the original draft writing, and approved the final version of the manuscript.

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Table 1: Overview of studies on in vitro culture of late secondary and EAFs in sheep

	Monte et al., 2019	Luz et al., 2012	Kamalamma et al., 2015	Cadoret et al., 2021	Barros et al., 2020	Barros et al., 2018	Barros et al., 2020	Arunakumari et al., 2010	Pathipati et al., 2019	Kona et al., 2021	Reddy et al., 2021	Silva et al., 2021	Menezes et al., 2019	Santos et al., 2019	Menezes et al., 2017	Tamilmani et al., 2005	Arunakumari et al., 2007
Source of ovaries	Sheep	Sheep	Sheep	lamb	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep
In vitro maturation results																	
Maturation rate [a] (%)	69 MI	29.63 MII	27.4 MII	56 MII	10.53 MII	31.5 MII	5.88 MII	~ 60 MII	16 MII	~ 38.8 MII	15 MII	14.29 MII	~ 20 MII	0 MII	7.15 MII	11 MII 30 MII	15 MII 55 MII
Culture conditions																	
Follicle diameter (µm)	250–300	>200	250 - 400	160-240	295-330	≥ 200	400-500	250 -400	250 -400	250–400	250–400	240- 260	300-450	200-230	200-233	150-250 251-400	150-250 250-400
Medium volume (µl)	100	100	20	100	100	100	100	20	80	20	-	100	100	100	100	10-20	20
Mineral oil	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	yes	Yes	-	Yes	Yes	Yes	Yes	yes	Yes
Concentration of gases	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2	5% Co2
Culture dish	60 mm plastic dishes	60 mm plastic dishes	35 mm plastic dishes	Petri dish	60 mm plastic dishes	60 mm plastic dishes	60 mm plastic dishes	35 mm plastic dishes	35 mm plastic dishes	35 mm plastic dishes	-	60 mm plastic dishes	60 mm plastic dishes	60 mm plastic dishes	60 mm plastic dishes	35 mm plastic dishes	35 mm plastic dishes
Culture system	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D	2D
Follicle isolation	Microdissection	Microdissection	Microdissection	Enzymatic-mechanical	Microdissection	Microdissection	Microdissection	Microdissection	Microdissection	Microdissection	Microdissection	Microdisection	Microdissection	Microdissection	Microdissection	Microdissection	Microdissection
Culture period (days)	18	18	6	20	18	18	12	6	6	6	6	12	12	12	12	6	6
Duration of medium replacement and volume	Every 2 days-60µl	Every 6 days-100µl	Every 6 days-10µl	Day 6 and 13-50µl	Every 2 days-60µl	Every 2 days-60µl	Every 2 days-60µl	Every 2 days-10µl	Every 2 days-40µl	Every 2 days-10µl	-	Every 2 days-60µl	Every 2 days-60µl	Every 2 days-60µl	Every 2 days-60µl	Only on day 3	-
Base culture medium	α-MEM	a-MEM	TCM 199	α-MEM	α-MEM	α-MEM	a-MEM	TCM199	TCM199	TCM199	TCM199	α-MEM	TCM199	α-MEM	α-MEM	TCM199	TCM199
Glutamine (mM)	2	2	-	2	2	2	2	-	-	-	-	2	2	2	2	-	-
Hypoxanthine (mM)	2	2	-	2	2	2	2	-	-	-		2	2	2	2	-	-
Hormones																	
Insulin***	10 ng/mL	10 µg/mL	-	6.25 µg/mL	10 ng/mL	10 ng/mL	10 ng/mL	-	-	-	-	10	10	10	10	-	-
GH(mIU/mL)	-	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-	-
FSH (ng/ml)	750 hrFSH	100-500 - 1000	-	100 - ovine FSH	-	750 hrFSH	-	2 (µg/mL)	2.5 µg/mL	2 (µg/mL) oviFSH	2 (µg/mL)		100 hrFSH	-	-	2 (µg/mL) Porcine FSH	2** (µg/mL)
LH (µg/mL)	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	1-2-4-8*	1
Melatonin***	-	-	-	-	1000	-	500	-	-	-			-			-	-

(pg/mL)																	
Leptin (ng/mL)	-	-	10	-	-	-	-	-	-	-	-	-	2	-	-	-	
Thyroxin (µg/mL)	-	-	-	-	-	-	-	1	1	1	1		-	-	-	-	1**
Estradiol-17 β (ng/ml)	-	-	-	-	-	-	-	-	-	5	5		-	-	-	-	-
Growth factors																	
TGFα (ng/ml)	-	-	-	-	-	-	-	-	-	-	-		-	-	-	2.5	2.5
EGF (ng/mL)	-	-	-	-	-	-	-	-	-	10**	-	-	-	-	-	-	
GDF-9 (ng/mL)	100	-	-	-	-	-	-	-	-	10**	-	-	-	-	-	-	
FGF (ng/ml)	-	-	-	-	-	-	-	-	-	10**	-	-	-	-	-	-	
EGF (ng/mL)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	50
LIF (ng/mL)	-	50	-	50	-	-	-	-	-	-		-	-		-	-	-
linoleic acid (µg/mL)	-	-	-	5.35	-	-	-	-	-	-	-	-	-	-	-	-	-
IGF-I (ng/mL)	50	-	-	-	-	-	-	10	10	10	10	-	-	-	-	-	-
Antioxidants																	
Transferrin (µg/mL)	5.5	5.5	-	6.25	5.5	5.5	5.5	-	-	-	-	5.5	5.5	5.5	5.5	-	
Selenium (ng/mL)	5	5	-	6.25	5	5	5	-	-	-	-	5	5	5	5	-	
Ascorbic acid (µg/mL)	50	50	-	50	50	50	50	-	-	-		50	50	50	50	-	
Gallic acid (µM)	-	-	-	-	-	-	-	-	-	-	-	100	-			-	
Kaempferol (µM)	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Protocatechuic acid (µg/ml)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	56.25	-	-

*LH at all concentration induced degeneration of all PFs

**Indicate the best concentration but not necessary combined with other additives

*** shows antioxidant activity

[a] Indicate the best MII rate which they were able to achieve.

Figures captions:

Fig 1: Schematic sequence of sheep follicular development.

Fig 2: Chromatin configuration during transition from secondary to the EAF stage.

NSN pattern, chromatin diffused in the nucleus without any sign of condensation.
SN pattern, condensed chromatin surrounded the nucleolus envelop.
SNE pattern, the condensed chromatin appeared localized partly around the nucleolus and partly close to the nuclear envelope. Oocytes are stained with the Hoechst 33342 Dye. Scale bar = 10 µm.

Highlights

1. Limited follicle availability and labor-intensive isolation methods hinder successful in vitro culture.
2. Overcoming diffusion limitations and providing optimal support for EAF growth and architecture are among the challenges of this system.
3. Novel isolation methods, bioengineering technologies, and supplementation with specific substances could be a promising approaches for improving EAF cult

Figure 1

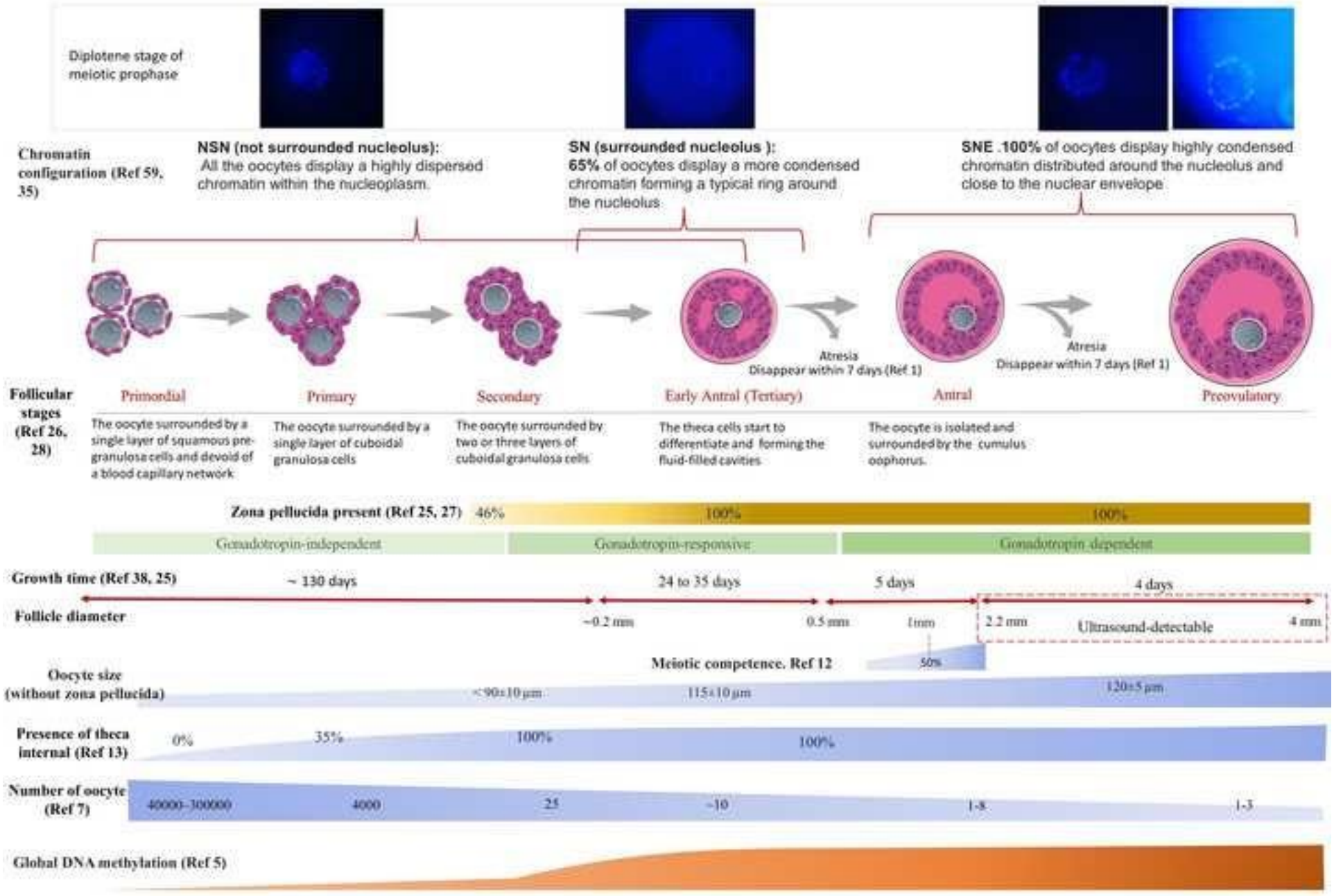

Figure 2