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Use of assisted reproductive technologies (ARTs) to shorten the generational interval in ruminants: current status and perspectives

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ABSTRACT

The challenges posed by climate change and increasing world population are stimulating renewed efforts for improving the sustainability of animal production. To meet such challenges, the contribution of genomic selection approaches, in combination with assisted reproductive technologies (ARTs), to spreading and preserving animal genetics is essential. The largest increase in genetic gain can be achieved by shortening the generation interval. This review provides an overview of the current status and progress of advanced ARTs that could be applied to reduce the generation time in both female and male of domestic ruminants. In females, the use of juvenile *in vitro* embryo transfer (JIVET) enables to generate offspring after the transfer of *in vitro* produced embryos derived from oocytes of prepubertal genetically superior donors reducing the generational interval and acceleration genetic gain. The current challenge is increasing *in vitro* embryo production (IVEP) from prepubertal derived oocytes which is still low and variable. The two main factors limiting IVEP success are the intrinsic quality of prepubertal oocytes and the culture systems for *in vitro* maturation (IVM). In males, advancements in ARTs are providing new strategies to *in vitro* propagate spermatogonia and differentiate them into mature sperm or even to recapitulate the whole process of spermatogenesis from embryonic stem cells. Moreover, the successful use of immature cells, such as round spermatids, for intracytoplasmic injection (ROSI) and IVEP could allow to complete the entire process in few months. However, these approaches have been successfully applied to human and mouse whereas only a few studies have been published in ruminants and results are still controversial. This is also dependent on the efficiency of ROSI that is limited by the current isolation and selection protocols of round spermatids. In conclusion, the current efforts for improving these reproductive methodologies could lead toward a significant reduction of the generational interval in livestock animals that could have a considerable impact on agriculture sustainability.

1. Introduction

In the last 30 years, animal breeding has been facing perhaps the most serious challenges since the so-called green revolution [1] trying to balance, on one hand, the increasing demand of food to feed an ever-growing world population [2], on the other, the need for a rapid adaptation to global warming, and for meeting the new selection criteria that favor animal wellbeing and disease resistance [3]. To meet these challenges, in livestock breeding programs, genomic selection (GS) has been widely used to increase the rate of genetic gain, improving livestock production efficiency and ultimately the sustainability of animal agriculture. GS has the advantage to shape modern breeding programs by using genomic information to estimate breeding values and rank selection candidates [4–6]. Over traditional phenotype-based selection, GS takes into account any trait that is recorded in the reference population. This approach allows to obtain increased selection intensity and

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greater selection accuracies, not limited to sex [7,8]. When GS strategies are used in combination with ARTs, the largest increase in genetic gain is obtained by shortening the generation interval [9]. This is possible since the convergence of GS, multiple ovulation embryo transfer (MOET), ovarian stimulation, ultrasound-guided transvaginal ovum pickup (OPU), *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* embryo production (IVEP), gives the opportunity to increase the female selective pressure reducing the generation interval within a timeframe of approximately one year [10]. Among ARTs, MOET allows a heifer to produce multiple living offspring only a few months after her first estrous cycle and can be performed every 6–8 weeks [11,12] whereas OPU can be performed every 2 weeks, further increasing the number of embryos produced per oocyte donor [13,14].

Further reductions in the generation interval can be achieved by using an advanced ART known as juvenile *in vitro* embryo transfer (JIVET) to produce offspring after the transfer of *in vitro* produced embryos derived from oocytes of prepubertal animals. However, the efficiency of this technique is still unsatisfactory, and more improvements are required before it can be used on a large-scale level. The greatest limitation of JIVET is the lower production of embryos compared to embryo development of oocytes from adult females. Furthermore, the new frontier of creating female and male functional gametes *in vitro* from neonatal gonads or embryonic cells is laying the foundations to an *in vitro* breeding approach that could shorten the generational intervals to a few weeks [15–17]. This includes the use of immature cells, such as round spermatids, for intracytoplasmic injection (ROSI) into IVM oocytes from adult and prepubertal animals that could remarkably shorten the generation time on the paternal side.

Aim of this review is to outline the current status, progress and potential in the use of advanced ARTs which can be applied to shorten the generational interval in both female and male of domestic ruminants. In particular, in the female section, we will present an overview of strategies for improving juvenile IVEP with regards to IVM systems of oocytes recovered from prepubertal ruminants. In the male section, we will discuss the methodologies to obtain the spermatogenesis *in vitro*. We will then focus on the methodologies used to isolate and characterize round spermatids, including their application to produce embryos *in vitro* by their injection into matured oocytes. In both cases, we will discuss the potential directions for future studies.

2. Reproductive biotecnologies to shorten the generational interval in the female

Particular interest has been focused on the IVEP by fertilizing oocytes recovered from prepubertal females. Following transfer of these embryos into adult recipient animals, offspring can be obtained from valuable animals before they reach sexual maturity, thereby reducing the generation interval and speeding the rate of genetic improvement. Normally, in ruminants, traditional MOET schemes result in a generational interval of about 12 months. Using oocytes obtained from 3- to 4-week-old donors it can be reduced this generation gap to 6 months only. Although the birth of lambs and calves has been already documented, the efficiency of juvenile IVEP is still low and variable [18–20]. Therefore, the current challenges are focused on improving the use of this technique. The limitations that still exist and new approaches to enhancing IVEP outcomes are discussed in the sections below.

2.1. *In vitro* embryo production from prepubertal donors

The procedure commonly applied to generate embryos from prepubertal derived-oocytes (Fig. 1) in domestic ruminants [21] includes different phases: i) the collection of oocytes from living unstimulated or hormonal stimulated donors (through vaginal or laparoscopic ovum pickup) or from slaughterhouse ovaries; ii) the selection of good quality oocytes and their culture under specific condition for IVM; iii) co-incubation of matured oocytes with *in vitro* capacitated spermatozoa for IVF; iv) *in vitro* culture (IVC) of presumptive zygotes up to the blastocyst stage. The use of prepubertal animals as oocyte donors allows to take advantage of the large pool of oocytes present in the ovaries of young animals for IVEP in livestock breeding programs to accelerate the propagation of superior, valuable animals. Indeed, a larger number of oocytes can be collected from prepubertal donors compared to their adult counterpart [22–24]. Despite this potential, juvenile IVEP is not yet efficient. The key factor limiting the success of IVEP is the poor developmental competence of prepubertal oocytes. Although species-specific variations in oocyte competency exist, oocytes collected from prepubertal domestic ruminants typically yield a lower blastocyst rate when compared with that obtained using oocytes from adult animals (10–30 % versus 40–60 %) [23,25–29].

Multiple studies underlined that different factors may influence the IVEP outcome including experiments and laboratory procedures along with the oocyte source (i.e. slaughterhouse or *in vivo* derived), hormone stimulation of donors before oocyte collection, donor's age [24,30–32]. Hormone stimulation prior to oocyte collection has been applied in prepubertal domestic donors to increase both the size of ovarian follicles and the number of follicles appropriate for aspiration and to enhance oocyte developmental *in vitro* [30,32–39]. Our experience [32] as well as results from multiple studies [27,40] indicate no variations in the *in vitro* developmental capabilities of oocytes from stimulated and unstimulated prepubertal ewes at 3–6 week of age. Moreover, the ovarian response upon gonadotropin stimulation was widely variable among prepubertal ruminants [41,42]. Plasma concentrations of anti-Müllerian hormone (AMH), a glycoprotein expressed by granulosa cells of small antral follicles, have been suggested as a good predictive marker of the ovarian response to gonadotropin treatment and *in vitro* embryo production in prepubertal heifers and lambs [43,44]. Hormonal stimulation regimes, as well as benefits and drawbacks, have been previously described in prepubertal domestic ruminants [33,39,45]. Therefore, this review will not address that topic.

Numerous studies examined the relationship between the age of prepubertal donors and the ability of the oocytes to develop, showing that, as the prepubertal donor approaches puberty, the oocytes ability to

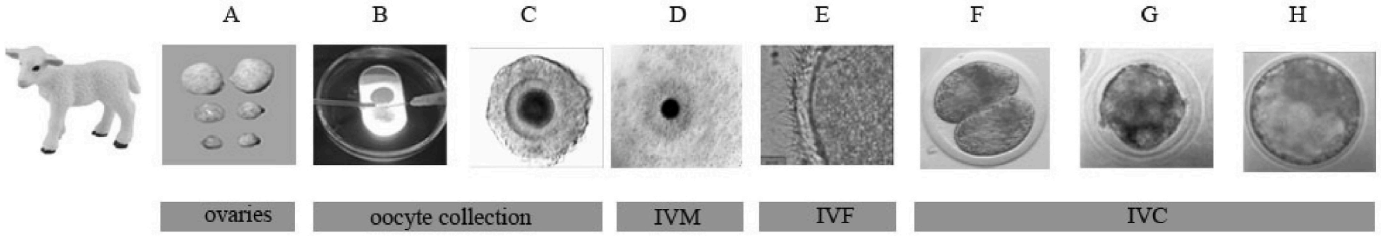

Fig. 1. Schematic sequence of steps in IVEP from prepubertal ewes (30–40 days old): ovaries with different morphology obtained from slaughtered lambs (A), ovarian slicing for oocytes collection (B); *in vitro* maturation (IVM) of oocytes cultured for 24 h: cumulus-oocyte complex (COC) (C), cumulus expansion after 24 h of incubation with gonadotropins (D); *in vitro* fertilization (IVF) with frozen-thawed ram semen (E); *in vitro* culture (IVC) of zygotes for 7 days: 2 cell-stage embryo (F), morula (G) and blastocyst (H).

respond to hormone stimulation and to develop increased [24,40,46].

Among the multiple steps of IVEP, IVM is the most crucial, because it is when oocytes acquire the potential to be fertilized and to sustain subsequent embryonic development. The goal of IVM is to support the complex process involving both the progression of the meiotic cycle and the reprogramming of cytoplasmic events which are necessary for the acquisition of the oocyte developmental competence [47]. The overall results in domestic ruminants indicated that prepubertal oocytes were able to reach the metaphase II stage at high rate (range 70–90 %) following 24 h IVM [48–50]. However, working with ovine oocytes, we found that the kinetic of maturation differs between adult and prepubertal oocytes, as well as prepubertal oocytes showed higher rates of spontaneous parthenogenetic activation [25,51]. On the other hands, with regards to cytoplasmic maturation, numerous studies evidenced that *in vitro* matured prepubertal oocytes had structural [52–55], biochemical [25,56,57] and molecular [58–66] abnormalities that are likely responsible for their decreased ability to undergo further embryo development. According to our research, *in vitro* matured prepubertal ovine oocytes differed significantly in their cytoplasm. In particular, compared to their adult counterparts, lamb oocytes showed altered distribution and activity of mitochondria [25,53], lower activity of the Mitogen-Activated Protein Kinase (MAPK) and the Maturation Promoting Factor (MPF) which could be responsible for the delayed kinetics of maturation and the high parthenogenetic activation [67]. Furthermore, we found that prepubertal oocytes had fewer and less functional transzonal projections (TZPs) [53,67], which are responsible for maintaining bidirectional communication between oocytes and cumulus cells (Fig. 2). These findings could indicate a reduced passage of molecules between somatic and germinal compartments, which is likely the cause of the prepubertal oocytes' poor metabolic and molecular condition [67,68]. Indeed, amino acid uptake and protein synthesis deficiencies [25,68] such as the expression of several genes related to metabolism and structural functionality were altered in prepubertal lamb oocytes compared to the adult counterpart [61].

Another significant difference between prepubertal and adult oocytes is their lipid content. Fatty acids are stored as triglycerides within different sized lipid droplets (LDs) that are located throughout the entire cytoplasm and play an essential role in oocyte development providing an endogenous energy reservoir [69]. Abazarikia et al. [70] described difference in characteristics and changes in the number and distribution of intracellular LDs in young and adult ovine oocytes. A lower LDs number has been reported in heifer vs cow oocytes after IVM [71]. Lipid accumulation is more evident in *vitro* than *in vivo* matured oocytes [72] and high-quality oocytes have high oleic acid levels, while low-quality oocytes have high stearic acid levels [73]. Variations of lipid contents in prepuberal oocytes, compared to adult, could be indicative of their importance in oocyte quality. Further work is warranted to understand the relation between lipids content and oocyte developmental competence and to explore the potential for utilizing IVM media supplemented with nutrients to increase the accumulation of lipids, thus supporting the successful maturation of prepubertal oocytes.

2.2. *Emerging approaches for improving in vitro maturation and competence of prepubertal oocytes*

The two main factors influencing the success of IVEP are the intrinsic quality of the oocytes and the culture systems for IVM [74]. A better understanding of the differences between oocytes from adult and prepubertal animals, the development of assays for the identification of competent oocytes, and the optimization of culture condition during

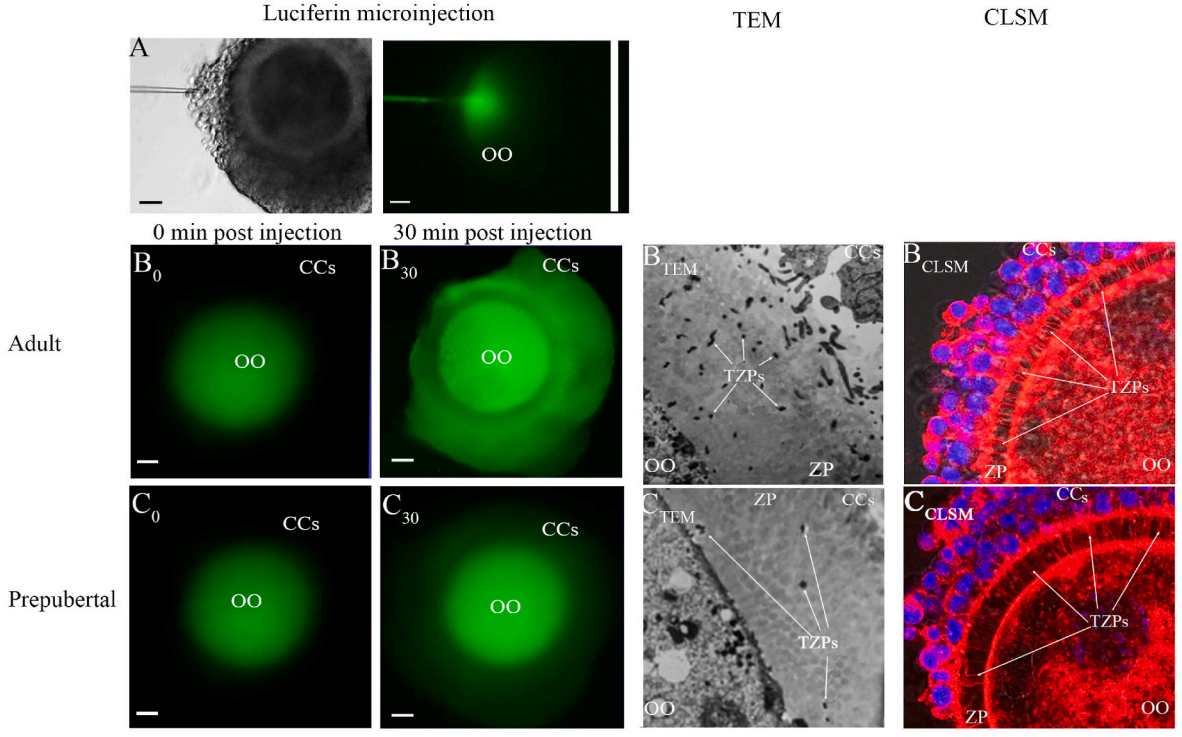

Fig. 2. Functional and structural intercellular communications in the cumulus–oocyte complexes (COCs) of adult and prepubertal ovine oocytes. Injection of the fluorescent dye Lucifer Yellow (LY) into oocyte cytoplasm by a microneedle (A). Junctional diffusion of LY from oocyte to cumulus cells (CCs): immediately after the injection the fluorescent dye is diffused in the oocyte cytoplasm in both adult (B_0) and prepubertal (C_0) COCs. After 30 min of culture LY is diffused into CCs compartment in the adult (B_{30}) while no diffusion was observed in the prepubertal CCs (C_{30}). Transmission electron microscope (TEM) section of adult (B_{TEM}) and prepubertal (C_{TEM}) oocytes for examination of transzonal projections (TZPs) of CCs through the zona pellucida (ZP). Laser scanning confocal microscopy (CLSM) images illustrating a detail of adult (B_{CLSM}) and prepubertal (C_{CLSM}) immature COCs following staining for F-actin (Rhodamine phalloidin, red), DNA (Hoechst 33358, blue). Transzonal actin fibers are visible in TZPs connecting CCs to the oocyte (OO). Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

IVM are all essential to improve the juvenile IVEP outcome. In the sections below we discuss *in vitro* approaches for identifying competent oocytes and for developing a customized IVM system to enhance the developmental competence of prepubertal oocytes from domestic ruminants.

2.2.1. Non–invasive methods of oocyte competence assessment

Traditional methods for selecting oocytes for IVEP programs are based on morphological criteria, including ovarian morphology assessment during the collection of oocytes from slaughterhouse material, follicle selection based on size, evaluation of COC morphology, (i.e. number and appearance of cumulus layers, oocyte size, cytoplasmic features by light microscopy [52,75–78]. However, morphological evaluation is not sufficient to predict oocyte competence. In recent years, high-throughput molecular technologies and novel non-invasive methods have been used for screening the ideal oocytes. Studies that performed molecular analyses of follicular fluid (FF), proteomic and transcriptomic evaluation of cumulus/granulosa cells and identification of biochemical components in oocytes have shown promising results.

2.2.1.1. Follicular fluid. FF provides an important microenvironment for oocyte growth and maturation and its composition varies according to physiological status of follicles and reflects oocyte quality [79,80]. FF is easily available during oocyte collection and it can be analyzed for identifying biochemical markers of oocyte quality [81,82]. Research has been performed to detect FF proteins in adult ruminants using various proteomic techniques [83–85]. Several FF proteins have been shown to affect the oocyte developmental competence [86,87]. The FF of prepubertal animals showed downregulation of several proteins associated with follicular development and oocyte competence [88,89], indicating the significance of proteomics in identifying markers associated with the low developmental competence of prepubertal oocytes. A metabolomic approach revealed numerous compounds in prepubertal FF indicators of the oocyte quality. For instance, a high Estradiol (E2) to Progesterone (P4) ratio in FF was related to a higher ATP content and a higher developmental competence in prepubertal sheep [90]; a lower FF glucose concentration and a high fatty acids concentration have been linked to low developmental competence of prepubertal goat oocytes [91–94].

Besides proteomic and metabolomic FF analysis, spectroscopic techniques such as Fourier transform infrared spectroscopy and Raman spectroscopy [95] may help to profile biochemical FF fingerprints [96] and to identify differences in FF between adult and prepubertal animals.

Also microRNAs (miRNAs), contained in FF extracellular vesicles (EVs) [97], has been shown to play a role in the follicle development [98] by regulating the expression of several genes in follicular cells [59, 99–102]. Therefore, several miRNAs differentially expressed in FF of adult and prepubertal ewes have been proposed as markers of oocyte quality [103]. Da Silveira et al. [104] demonstrated that the supplementation of culture media with EVs isolated from FF increased blastocyst rates in cattle. Therefore, it is possible that adding EVs derived from adult FF to IVM medium of prepubertal oocytes may enhance IVEP outcome.

Collectively, these types of analyses constitute a key step in identifying the variation in FF composition between prepubertal and adult stage as well the specific substances or factors that may have beneficial impacts on oocyte competence. These insights could help design specialized IVM conditions that better meet the needs of prepubertal oocytes and improve nuclear and cytoplasmic maturation, fertilization and embryo development to the blastocyst stage. This topic will be discussed in the paragraph 2.2.3.

2.2.1.2. Cumulus cells. CCs surround the oocyte, and the bidirectional cross-talk between these compartments modulates the development of both cell types [105,106]. Changes in oocyte developmental competence affected cumulus cell phenotype and gene expression [107]. The analysis of CCs, which are normally discarded during IVF treatments, allows for the non-invasive evaluation of molecular markers that affect the quality and developmental potential of oocytes. Increased CCs apoptosis is a predictive indicator of impaired oocyte maturation, fertilization [108], preimplantation embryo development [109,110] and reduced pregnancy outcome after IVF [111]. The high incidence of apoptosis in CCs of prepubertal goat oocytes has been linked to the lower development to blastocyst stage compared to the adult counterpart [112]. The prognostic significance of the CC apoptotic rate in predicting the oocyte quality and outcome of IVF and embryo transfer, however, is debated [108,113].

Transcriptomic approaches can be used to assess the gene expression level in follicular cells to identify oocyte competence-predictive molecular markers and to clarify their functional significance [114]. Changes in the gene expression of CCs and granulosa cells have been linked to a variety of outcome parameters, including *in vitro* embryo development and pregnancy [115–118]. Hundreds of genes and proteins with differential expression between adult and prepubertal sheep were discovered by transcriptomic and proteomic analysis of CCs and granulosa cells [119–121]. These genes and proteins were connected to a number of pathways, including hormone biosynthesis, cell-cell adhesion, the insulin-like growth factor pathway, and embryo development [49]. Many of them have been selected as markers of developmental competence in lambs [107,122]. These findings indicate that there are notable differences in gene expression and protein patterns between prepubertal and adult CCs and granulosa cells, which partially explain the reasons for the reduced development competence of prepubertal oocytes. IVM approaches based on co-culturing prepubertal oocytes with adult granulosa/CCs or their supernatant may help to enhance oocyte developmental competence. Furthermore, bioengineering techniques designed to mimic physiological follicular structure (see section 2.2.4) may be used as a means of improving the CCs function and promoting interactions between the somatic and germinal compartments, both of which can enhance the IVEP outcome.

2.2.1.3. Oocyte. Several attempts have been made to develop non-invasive techniques that can be combined with the conventional morphological selection to predict the structural, molecular, and biochemical characteristics of oocytes [102,123]. The measurement of oocyte metabolism by the analysis of spent oocyte culture medium has been proposed to select oocytes with high developmental capacity. Indeed, several oocyte metabolism-related factors, such as ammino acid turnover, glucose, oxygen, and pyruvate have been evaluated and linked to oocytes quality [124–127]. Novel methods such as hyperspectral microscopy, laser confocal imaging, and fluorescence lifetime-imaging microscopy [128–136] have recently been applied to the optical measures of the oocyte metabolic signature. Furthermore, vibrational microspectroscopies such as Raman microspectroscopy and Coherent anti-Stokes Raman scattering microscopy have been effectively used to investigate the global biochemical profile of mammalian female gametes and to identify at sub cellular level structural and molecular features that are associated with the quality of the oocytes [128–141]. To date, there have been no reports of using these techniques on prepubertal oocytes. These techniques involve expensive and sophisticated equipment, and more research is necessary to determine whether they are safe to use on reproductive cells [134,142].

A simple method that proved to have predictive potential for oocyte quality evaluation is the measurement of glucose-6-phosphate dehydrogenase (G6PDH) activity using brilliant cresyl blue (BCB) staining. BCB is a blue compound which is reduced by G6PDH in a colorless substance [143]. G6PDH activity is high in growing oocytes and decreases in fully grown oocytes. As a result, the oocytes that have finished the growth phase are blue (BCB+), whereas the oocytes that are still growing are colorless (BCB-). This technique has been used for the

selection of immature oocytes before IVEP in various species [144]. Goat and sheep prepubertal oocytes' G6PDH activity was assessed by BCB staining, which revealed that BCB+ oocytes had a greater capacity for development [145–147]. In a previous study, we found that the integration of the BCB test and the addition of resveratrol in the protocol of IVEP improved the blastocyst production from prepubertal lamb oocytes [50].

These techniques may be useful in determining the molecular composition of the oocyte in relation to its developmental potential. Combining the morphological classification with the easy-to-use and low cost BCB test can be an effective method in the IVEP procedure for differentiating oocytes with good and poor developmental competence. This will enable to apply IVM conditions that are specifically tailored to the oocyte quality.

2.2.2. Pre-maturation culture of oocytes to enhance cytoplasmic maturation

At the time of collection for IVEP, prepubertal oocytes are still in the process of acquiring developmental competence within growing antral follicles. Intra-oocyte levels of cyclic adenosine 3,5 -monophosphate (cAMP) and cyclic guanosine 3,5 -monophosphate (cGMP), two key regulators of oocyte meiotic maturation [148] decrease when oocytes are removed from their follicles, causing the spontaneous resumption of meiosis and desynchronization between nuclear modification and structural/molecular cytoplasmic changes which are fundamental for the acquisition of the developmental competence [149]. A short 'pre--maturation' (Pre-IVM) culture period in presence of meiotic inhibitors prior to IVM might help to synchronize oocyte nuclear and cytoplasmic maturation stages [150]. So far, this approach, also known as bi-phasic maturation, has been successfully applied in IVEP programs in adult domestic ruminants [57,151–161]. Several pharmacological and physiological agents (cGMP/cAMP modulators, inhibitors of phosphodiesterase, proteins synthesis and MPF) were tested in various pre-IVM systems to modulate intra-oocyte cAMP/cGMP concentration and to control meiotic arrest and resumption [148,150]. To date, only few studies explored the potential of pre-maturation culture in juvenile IVEP. A biphasic IVM, including a pre-IVM with c-type natriuretic peptide (CNP), the physiological meiosis-inhibiting regulator [162], plus estradiol (E2) allowed maintaining meiotic arrest for 6 h in prepubertal goat oocytes, improved the oocyte protection against oxidative stress, up-regulated genes related to DNA methylation and extracellular matrix formation while maintaining cumulus-oocyte communication and enhanced the embryo developmental competence to the blastocyst stage after IVF (CNP + E2: 29.9% vs control: 18.1 %) [163]. Similarly, pre-IVM of lamb oocytes in media containing CNP and E2 and subsequent standard IVM improved the percentage of blastocysts developed (CNP + E2: 44.0 % vs control: 32.6 %) [164]. In another study, Wang et al., 2016 found that culturing low quality lamb oocytes (BCB- selected oocyte) with a phosphodiesterase 3 (PDE3) inhibitor, milrinone, yielded higher proportion of blastocysts (25 %) than the control system (2.7 %) [165].

These results, although preliminary, indicated that pre-IVM is a promising step that might be included to IVEP schemes when using oocytes from prepubertal animal. Additional research should be conducted to extend the transient meiotic arrest for more than 6–8 h and to implement the pre-IVM medium with other meiotic-inhibitors and other factors such as hormones and growth factors, which could further improve the competence of prepubertal oocytes. In prepubertal mice, the blastocyst rate of oocytes pre-IVM for 24–28 h with CNP in medium containing FSH and GDF9 was similar compared to IVEP of ovulated oocytes [166].

2.2.3. Formulation of tailored IVM culture media

Culture conditions for IVM of prepubertal oocytes have long been developed based on those typically used for adult oocytes. Recently, thanks to the knowledge gained from studies on FF, CCs and oocytes, various attempts have been devoted to design specific culture media for IVM of prepubertal oocytes to better meet their need and to ameliorate cytoplasmic maturation, proper fertilization, and embryo development to the blastocyst stage. In domestic ruminants, a variety of compounds have been added to the culture medium for IVM of prepubertal oocytes, including antioxidants, cytokines, and growth factors, either alone or in combination. In the following paragraphs and in Table 1 we summarized the results obtained in recent years with the use of the most effective additives in IVM media for enhancing IVEP in prepubertal domestic ruminants.

2.2.3.1. Antioxidants supplementation to counteract oxidative damage.

One of the well-known key factor which may contribute to the overall poor quality of *in vitro* matured oocytes is oxidative stress (OS) which generates from an imbalance on the production and elimination of intra-oocyte reactive oxygen species (ROS) and causes damage to oocyte structures [167]. This issue is considerably more relevant for prepubertal oocytes because they are less able to maintain an appropriate redox homeostasis in response to OS generated by the *in vitro* condition compared to those from adult oocytes. This may be caused by the impaired synthesis of endogenous antioxidants in prepubertal oocytes [168,169] like glutathione (GSH) that is the main non-enzymatic defense system against oxidative stress in oocytes [170]. Due to its ability to increase cysteine uptake and to promote intra-oocyte GSH synthesis, low molecular weight thiol such as cysteamine is regarded as the standard antioxidant in the IVM of adult animal oocytes [171]. Beneficial effects of the addition of cysteamine to the IVM medium have been reported in IVEP from prepubertal goats and lambs [146,172,173]. Other antioxidants used in IVM of adult oocytes in various species have been tested to attenuate the deleterious effect of OS on the developmental competence of prepubertal oocytes, including hormones, and compounds of natural origin.

Melatonin (N-acetyl-5-methoxytryptamine), an indoleamine synthesized in the pineal gland and other organs such as the ovary, deserves special attention due to its powerful free radical scavenger activity and its wide-ranging antioxidant action [174]. It has been demonstrated that melatonin supplementation during IVM reduced ROS levels and enhanced mitochondrial activity and ATP content in prepubertal goat oocytes [175] and increased rate and cell number of blastocysts in both prepubertal goats and calves [175,176]. On the other hand, Tian et al. [177] did not find positive effect of melatonin supplementation during IVM on development rates of oocytes retrieved from 4–5-week-old lambs.

Due to their efficiency and low cost, using compounds of natural origin might also offer a good option to counteract deleterious effect of OS in prepubertal oocytes. Among them, resveratrol (Res), a non-flavonoid polyphenol naturally presents in several plants, such as nuts, mulberry and grapes is one of the most studied [178]. This compound functions as a chemical activator for mitochondrial biogenesis in addition to being a potent ROS scavenger [179]. In prepubertal goats, the supplementation of IVM with Res increased intra-oocyte GSH content, modified the mitochondrial distribution and lead to higher blastocyst yield than control IVM without the antioxidant [50]. The screening of higher intrinsic quality prepubertal goat oocytes (positive to the BCB stain, BCB+) and IVM in the presence of Res led to a further increase in development to the blastocyst stage. Our group (Bogliolo L. unpublished data) also evidenced a positive effect of Res addition during IVM of lamb oocytes resulting in enhanced embryo development up to the blastocyst stage (31.5 %) compared to non-treated group (15.7 %). In prepubertal bovine oocytes, Res treatment during IVM did not affected blastocyst yield while enhancing the percentage of expanded blastocysts [180].

Another interesting polyphenol is verbascoside (Vb) which is present in olive oil and can be obtained from the oil production wastewater [181]. Compared to other antioxidants which act at micromolar concentrations, Vb exerted positive effects at much lower levels. Indeed,

Table 1
List, concentrations, and effects of compounds that have been proven to enhance the developmental competence of oocytes when added to the IVM medium in domestic prepubertal ruminants in recent years.

Antioxidants	Species	Optimal Dose	Effect	Blastocyst rate vs (control)	REF
Melatonin	bovine	0.01 nM	↑blastocyst yeld/cell number	23.1 % (11.1 %)	[176]
	goat	0.1 μM	↑blastocyst yield/cell number	28.9 % (11.7 %)	[333]
Resveratrol	goat	1 μM	↑blastocyst yield	20.1 %(6.8 %)% 28.3 % (13.0 %)[a]	[50]
	bovine	1 μM	↑expanded blastocyst rate	63.8 % (42.8 %)[b]	[180]
	sheep	1 μM	↑blastocyst yield	31.5 % (15.7 %)	Bogliolo et al. (unpublished data)
Verbascoside	sheep	1 nM	↑blastocyst yield/cell number	20.5 % (13.2 %)	[182]
Sericin		0.5 %	↑blastocyst yield	31.2 %(15.1 %)	[177]
Cerium dioxide nanoparticles (CeO2 NPs)	sheep	44 μg/ml	↑blastocyst yield/cell number	22.8 % (7.0 %)	[186]
Cytokines/Growth factors					
Follicular Fluids	sheep	20 % FF from FSH stimulated sheep	↑blastocyst yield	31.4 % (20.7 %)	[189]
ITS + FLI	sheep	ITS: 1.0 mg/ml insulin, 0.55 mg/ml transferrin, 0.5 μg/ml selenium: FLI: 40 ng/ml FGF2, 20 ng/ml LIF, 20 ng/ml IGF1	↑blastocyst yield	34.9 % (18.5 %)	[177]

[a] BCB + selected oocytes.
[b] Percentage of expanded blastocyst/total embryos.

Martino et al. [182] documented that supplementation with Vb nanomolar concentrations during IVM improved blastocyst formation and quality by protecting lamb oocyte against OS. However, a Vb pro-oxidant activity during IVM of prepubertal ovine oocytes has been evidenced depending on concentration and exposure times [183].

Sericin, a water-soluble natural protein from the silkworm, with antioxidant action [184,185], supplemented during IVM significantly increased the rate of blastocyst obtained from lamb oocytes [177].

Beside the use of compounds of natural origin, engineered nanoparticles of cerium dioxide (CeO_2 NPs) have also been tested for their powerful redox activity. A low concentration of CeO_2 NPs in the maturation medium enhanced IVEP of prepubertal ovine oocytes and resulted in blastocyst rates comparable to those of adults [186]. However, the safety of these compounds needs to be extensively investigated before being used in IVEP programs. Overall, these findings may help to determine the most effective antioxidant and its appropriate concentration for improving prepubertal oocyte development competence.

2.2.3.2. Follicular fluid components: cytokines, growth factors, fatty acids. FF composition of prepubertal and adult animals differed [92,187,188]. The variations in FF composition may partially explain the lower developmental competence of prepubertal oocytes compared to adults' ones.

In this context, Tian and at [189]. performed a study maturing lamb oocytes in medium supplemented with 20 % adult FF from FSH-stimulated ewes or adult FF from abattoir-derived ovaries and yielded higher blastocyst rates than that from the control medium.

Other studies aimed at improving the developmental competence of prepubertal oocytes by supplementing IVM medium with cytokines and growth factors which are important component of FF and have fundamental regulatory effect on cumulus-oocyte complexes. Tian et al. [189] assessed the impact of adding the FLI cocktail, containing fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF), and insulin-like growth factor (IGF1), to IVM media for lamb oocytes and observed a significant increase in blastocyst formation. FA are other important components of the FF because they provide a significant source of energy for the developing and maturing oocytes [79]. The effect of omega-3 a-linolenic acid (ALA) addition to the IVM medium on embryo development of prepubertal sheep oocytes has been explored [190]. Although no differences were observed in blastocyst development, ALA treatment improved the quality of blastocyst improving their total cell number and reducing the number of apoptotic cells [190]. These improvements of the IVM conditions are particularly promising for further optimizing the efficiency of IVEP in prepubertal animals.

2.2.4. Bioengineering approaches that mimic the physiological follicle environment

In addition to identify the best chemical composition of the IVM culture media, reproducing the physiological follicular architecture and microenvironment is crucial to drive proper oocyte nuclear and cytoplasmic maturation.

Bioengineering strategies tailored to mimic physiological follicular structure have garnered a lot of interest in the recent years [191]. These approaches focused on i) preserving the cumulus oocyte complexes' three-dimensional (3D) structure to enable bidirectional communication between the oocytes and the surrounding granulosa cells; ii) establishing dynamic culture systems to allow fluid chemical compositions to change in order to mimic more accurately the *in vivo* environment; iii) recreating the composition of the follicular extracellular matrix to promote interactions between the COC and granulosa cells thus improving the efficacy of signaling pathways of oocyte maturation.

Specific culture methods and matrices have been developed for 3D IVM culture of oocytes in various species including agarose matrix [192], glass scaffolds [193] and alginate microbeads [194,195]. These techniques made it possible to avoid 2D culture disadvantages including COCs flattening at the bottom of the culture plate, which significantly reduced the amount of cell surface exposed to media and reduced the relationship between regulatory factors and their receptor sites, thus improving the efficacy of the signaling pathways that regulate meiotic maturation and improved blastocyst production. Recently, Mastrorocco et al. [194] developed an automated one-step bioprinting method for preparation of COC- alginate microbeads that was highly reproducible and capable of controlling cumulus size and integrity, addressing the limitation and variability of two-step or manual procedures mentioned in previous studies. This technique was successfully used for IVM of prepubertal ovine oocytes. Indeed, the 3D system supported oocyte nuclear maturation more efficiently than the 2D control, increased ooplasmic mitochondrial activity and ROS generation ability, up

regulated maternal-impact genes and improved blastocyst quality. The same group reported that the addition of granulosa cells and type I collagen in the alginate COC-microbeads or the culture of COC-microbeads under dynamic IVM conditions in a millifluidic bioreactor further improved lamb oocytes IVM [196,197]. Such methods more accurately mimicked the physiological follicle structure and flow conditions, which had a positive impact on the nuclear maturation and bioenergetic state of IVM lamb oocytes.

Another 3D strategy for oocytes IVM is based on the production of microbioreactor polytetrafluoroethylene (PTFE) micro-bioreactors, also known as liquid Marbles (LM) [198], which consists in a drop of IVM medium enclosed in hydrophobic PTFE confining the oocytes in a small space and encouraging them to freely interact with each other while, at the same time, allowing the gas exchange between the medium inside the reactor and that in the holding culture vessel. Bebbere et al. [199], recently, proved that LM provided a suitable environment for IVM of lamb oocytes as demonstrated by modulating the expression of oocyte-secreted factors such GDF9, of enzymes involved in DNA methylation reprogramming and of the subcortical maternal complex as well as increasing the blastocyst rate compared to the traditional 2D culture system. Preliminary studies of our group found that using LM for a biphasic IVM of lamb oocytes (pre-IVM with CNP and E2) prolonged the persistence of CCs-oocyte communications and reduced the OS (unpublished data).

3. Assisted reproductive technologies to shorten the generational interval in the male

Male puberty consists of physical changes leading to sexual dimorphism through the development of the secondary sex characteristics. This period has been correlated with the pulse and surge modes of gonadotrophin secretion, generated by the gonadotrophin-releasing hormone (GnRH) neuronal network [200].

In cattle, the achievement of male puberty is defined as the bull ability to produce an ejaculate containing 5.0×10^7 spermatozoa with at least the 10 % of spermatozoa characterized by progressively linear motility [201]. Several studies also correlated puberty with scrotal circumference, sperm quality (concentration, motility and morphology) and circulating blood concentrations of reproductive hormones with the timing of the early transient rise in Luteinizing hormone (LH) pulse being a critical factor in determining the age at which puberty is reached [202]. The average age of bull puberty is about 315 days, with a range of 292–327 days for beef bulls [203–205] and an overage of 320 days with a range of 283–369 days for dairy bulls [206–208]. Therefore, the generation interval could be shortened breeding males as early as possible after birth, or, even more, in case functional gametes could be obtained even before birth. It will be now discussed how this could be achieved and how far away is this goal in domestic ruminants and in cattle in particular.

3.1. Recreating spermatogenesis in vitro

Several studies tried to develop culture systems that allow spermatogenesis and spermiogenesis to occur outside the body. The various attempts were based on the use of both 2D and 3D culture systems. However, the results obtained so far show that it is challenging to recreate the entire process *in vitro* and only a partial differentiation has been achieved. In 2D culture systems, the co-culture of a feeder layer, of Sertoli or Vero cells, promoted the differentiation of SSCs and spermatocytes towards haploid spermatids [209–213] both in humans and mouse [214]. However, the differentiation of SSCs into mature spermatozoa has been rarely observed.

In cattle, 2D culture systems have been applied using the same approaches developed in humans and mouse. Several research groups reported that it is possible to isolate SSCs from bull testis and keep them alive in culture. It has also been shown that bovine spermatogonia form large colonies [215,216] in which groups of differentiating cells can acquire characteristics of haploid spermatids. The use of feeder layers has also been used in this species; however, the results are controversial. While some authors compared different feeder layers showing that STO cells are suitable for short-term propagation of bovine SSCs that maintained their ability to propagate as well as the expression of SSC major markers [217]. Oaetly and colleagues [218] developed a feeder-free system with bovine fetal fibroblast-conditioned medium that sustained bovine undifferentiated spermatogonia for at least one month *in vitro*.

While in cattle there is a high interest in developing these methodologies due to the economic impact of the applications that could derive, few studies have been reported also in other farm species such as pig [219], horse [220] and Mediterranean buffaloes [221]. In the latter, it was possible to isolate SSCs which survived, proliferated and differentiated towards elongated spermatids, characterized by morphological features of flagellum and expression of the PRM2 gene, but not by the expression of the TP1 genes [221].

Overall, although these studies indicate that 2D culture systems can only be used to obtain an incomplete spermatogenesis, they identified specific supplements such as bone morphogenic marrow 4 [222], epidermal growth factor [223], retinoic acid [224], rFSH [214], insulin transferrin selenium [225] that constitute the signaling mechanisms responsible for spermatogonia differentiation providing valuable insights for developing a fully defined stepwise *in vitro* spermatogenesis system. Several 3D platforms for *in vitro* spermatogenesis in humans and mouse have been developed to counteract infertility problems. One of these systems are the organotypic cultures developed for mimicking the natural microenvironment of the complete testicular niche [226]. An example is the hydrogel bioreactor made of a hollow chitosan cylinder [227]. However, one of the limitations of these systems is the difficulty to keep the tissues viable and functional without a bodily support, including delivery of oxygen, vitamins, nutrients and trophic factors through diffusion from the local vascular system. An alternative system is the bioengineering of the testicular niche [228]. This method is based on recreating the physiological tissue structure using 3D scaffolds, organoids or 3D bio-printed systems, to increase intercellular connections and diffusion of cell-secreted factors by recapitulating the tissue morphology. The advantage of this approach compared to organotypic culture systems is that it mimics not only the tissue organization, but also its cellular composition. Moreover, cell viability is improved by the nutrient and oxygen diffusion from the medium through porous scaffolding.

In cattle, a 3D scaffold was developed to culture and differentiate SSCs isolated from neonatal bull testis. After tissue dissociation, the different cell types, including spermatogonia and Sertoli cells, were reaggregated and encapsulated in calcium alginate [229]. This system improved the long-term culture conditions of germ cell differentiation compared to the results obtained with 2D systems when starting with the testis of a pre-puberal subject [229]. Another 3D testicular culture model uses a decellularized scaffold generated eliminating the cellular component of the tissue while preserving the extracellular matrix (ECM). The rationale of this approach is based on previous studies that demonstrated the effect of the ECM on the preservation and differentiation of various stem cells [230–233]. On this basis, Movassagh and colleagues [234], recently demonstrated that the culture of SSC cells on a decellularized sheep testicular matrix provided the conditions suitable for their preservation and proliferation.

The most advanced frontier in recreating spermatogenesis *in vitro* has seen the use of mouse pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) derived from somatic cells, to generate germ cells *in vitro* and differentiate them towards fully mature spermatozoa. Hayashi and colleagues [235] reported the generation of primordial germ cell-like cells (PGCLCs) in mice with robust capacity for spermatogenesis. PGCLCs were generated from ESCs and induced iPSCs through epiblast-like cells (EpiLCs), a cellular state highly similar to pre-gastrulating epiblasts but distinct from

epiblast stem cells (EpiSCs). However, in this work, they recreated spermatogenesis only partially. Ishikura and colleagues [236] were then able to derive spermatogonia-like cells from PGCLCs after their aggregation and subsequent culture with fetal testicular cells. In this work, the authors were able to further differentiate spermatogonia-like cells into spermatids which were used to obtain live and fertile offsprings not only following transplantation into testes *in vivo* but also culturing testis transplants *in vitro* [236]. Similarly, co-culture of PGCLCs with neonatal testicular somatic cells and addition to culture media of morphogenic and sexual hormones allowed to recapitulate male gametogenesis *in vitro* with resulting spermatid-like cell generation. Their intracytoplasmic injection into mature oocytes produced viable and fertile offspring [237].

Interesting results came from a study where male mice, pigs, and goats rendered genetically sterile by CRISPR-Cas9 editing of the NANOS2 gene supported donor-derived spermatogenesis following allogeneic stem cell transplantation [238]. In the same work, it was shown that CRISPR-Cas9 editing of the NANOS2 gene in cattle leads to male germline ablation. Collectively, these advancements represent a major step toward realizing the enormous potential of surrogate sires as a tool for dissemination and regeneration of germplasm in all mammalian species [238]. Similar results were also obtained in sheep where germ cell transplantation was successfully obtained between different rams of different breeds [239]. However, in this work, it was important to use a single dose of irradiation 6 weeks before transplantation for preparing recipient testis at a puberal stage [239].

Among domestic animals, stable ESCs have been established in cattle only. These ESCs showed a stable morphology and karyotype, and expression of pluripotency markers as observed for in mouse and human ESCs. However, the plasticity of ESC and iPS in cattle, despite some recent advances [240], is still much more limited than in mouse [241] thereby preventing the replication of such achievements. At present, these results have not been replicated in any other species. However, the results achievable by their application to bulls would be useful. If spermatogonia could be derived from embryonic or induced pluripotent stem cells, spermatozoa generated *in vitro* from embryos that, in turn, could generate other embryos. Repeating this cycle for a few times, the generation interval could be reduced to a few weeks. This could open the way for rapid improvement in a wide range of traits, especially low heritable traits that control key features like disease resistance and environmental adaptation.

3.2. How not to wait for puberty anyway

Even if the scenario described here it is still not applicable to ruminants, there is still a lot of work that can be done in order to bring it closer to reality. The first and realistic step to make possible the use of spermatids for fertilizing an oocyte bypassing the need for a full differentiation into a functional spermatozoon. The procedure is known as Round Spermatid Sperm Injection (ROSI) and is widely used in humans and mouse to overcome lack of a functional tail and led to the generation of full-term individuals in both species. Assuming that an IVF procedure followed by ESC establishment takes about 4 weeks in cattle, and germ cell differentiation takes about 2 or 3 months in mice, the use of *in vitro* breeding could be completed in around 3–4 months. Moreover, if spermatids could be used for fertilizing mature oocytes, this would mean a huge reduction in the generational interval. Notably, *in vitro* breeding might be associated with the modern techniques of GS to have a greater effect on genetic improvement in a reduced amount of time.

Unfortunately, in livestock species, on the contrary, ROSI although possible, it has a very low success rate, and no offspring have been born so far. It will be now analyzed the possible reasons for this difference and how we can overcome the problems.

3.2.1. The isolation of round spermatids

The methods for isolating round spermatids have been mainly developed in mouse and include velocity sedimentation, density gradient [200–202], centrifugal elution [242,243], immunoselection panning technique [244]. Among these, the use of Percoll density gradient, which is routinely applied to isolate motile spermatozoa, has been also applied in cattle to simultaneously separate the different cell types found in the seminiferous tubules, which include spermatogonia, spermatocytes and spermatids [245]. However, in cattle, Ock and colleagues [245] obtained only a total of 30–40 % of presumptive round spermatids on the total cells isolated, showing that the isolated round spermatids were contaminated by several other cell populations including Sertoli and somatic cells, while a pure round spermatid population was not obtained [245]. Recently, in mouse, Kim and colleagues [246] reported a simple method to isolate spermatid fractions from mouse testes using unit gravity sedimentation in a BSA density gradient. However, they too reported issues on the purity of the obtained cell population since each fraction contained several somatic and spermatogenic cell types [246].

Flow cytometry combined with cell sorting has been also used for isolating round spermatids. This method is based on the different DNA content of haploid spermatids compared to the other spermatogenic cells, such as spermatogonia and spermatocytes, which are characterized by a diploid genome [247]. In mouse, haploid round spermatids were isolated after staining with Hoechst 33342 or Dye Cycle Violet) or by cell diameter and granularity using flow cytometric cell sorting [248].

Recently, Simard and colleagues showed that using syto16, another intercalating DNA dye, it was possible to isolate not only a pure population of mouse spermatids but also to separate different spermatid stages using the variable fluorescence intensity [249]. The isolated spermatid population were characterized by a different nuclear reorganization. In this work, the authors hypothesised that the observed differences could be due to the different chromatin remodeling of the spermatids which occurs during the formation of their peculiar chromatin structure transition where histones are replaced by protamines [249]. Although the round spermatids isolated using this method cannot be used for ART applications, such as ROSI, due to the persistence of syto 16 within the DNA, these methodologies could be used to perform molecular analysis on the isolated spermatid populations to unravel the mechanisms and the molecular pathways at play during spermatogenesis. In conclusion, while in mouse and humans several methods have been developed for isolating pure populations of round spermatids, these approaches have not yet been fully applied to domestic animals including ruminants. Moreover, in all the mammals, the development of a minimally invasive selection of round spermatids may yield high purity populations for ARTs not only in humans and mouse but also in domestic animals as well.

3.2.2. Distinctive morphological features of round spermatids

Spermatid morphological features and size can be different among species [213,250]. The presence of nucleoli is a distinctive feature of round spermatids (Fig. 3). These are identified in all mammals, including humans [251] and mouse [252]. However, while in humans and mouse, spermatid diameter is from 6.0 μm to 11.0 μm [246,253], in cattle, it ranges from 7.6 to 13.4 μm [254,255].

Another typical feature characterizing round spermatid is the acrosome. Among species, acrosome size and shape can be different in the spermatozoa. In humans, the acrosome is very thin and closely wraps the nucleus. In rabbit the anterior margin is clearly evident [256]. Whereas, on the bull spermatozoa, the acrosome is characterized by a distinct smooth crescent shape of the apical ridge and a smooth surface [257].

Taken together, the heterogeneity of round spermatids may explain why it can be difficult to identify competent round spermatids to inject into the mature oocytes using only phase contrast microscopy.

Therefore, it is necessary to establish a criterion for their correct selection.

Reyes and colleagues [258] used karyotyping and fluorescence in

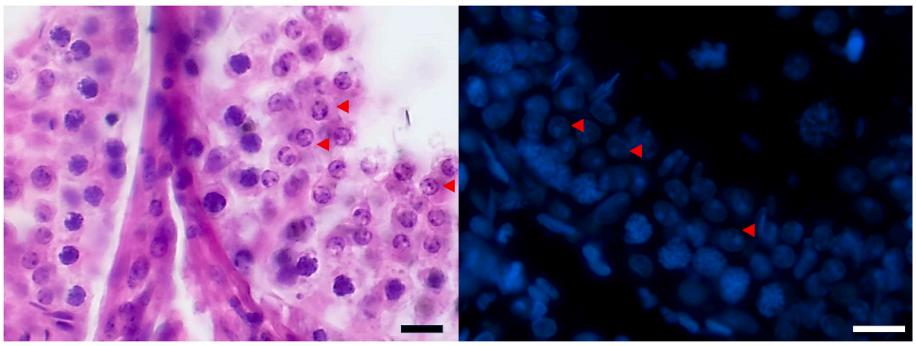

Fig. 3. Representative pictures showing cell and nuclear morphology of round spermatids (indicated with red arrows) in the seminiferous tubule of post-puberal bovine testis. The images were produced using 5 μm-thick histological sections of formalin fixed, paraffin embedded testicular tissues which were stained using hematoxylin and eosin (left side) or 4′,6-diamidino-2-phenylindole (DAPI, right side). Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

situ hybridization (FISH) to identify exactly the haploid spermatids, however this procedure is invasive and selected spermatids cannot be used for ROSI [258]. In 1999, Sutovsky and colleagues [259] demonstrated that bovine and rhesus round spermatids contain highly polarized mitochondria [259]. These results were also confirmed in humans where spermatogenic cells were categorized based on stage-specific mitochondrial location and morphologic change [260]. Following these studies, Hikichi and colleagues [261] used mitochondria polarization for identifying the different mouse spermatogenic cells. In particular, round spermatids mitochondria were distributed either across the whole cell or localized to the cell rim around the nuclear membrane [261]. In the same work the authors showed that, using this selection criteria, embryo developmental rate was the same when produced by ROSI and when produced by ICSI [261]. Although these studies suggest that mitochondria could have a possible role in determining the competence of round spermatids to fertilize the oocytes, a clear correlation with round spermatid quality has not been defined yet. However, all these methods are invasive as they are based on the use of a staining procedure. Therefore, this represents an impediment for applicability of these procedures to IVEP as they can affects spermatid quality and viability. As a consequence, they cannot be applied yet in the selection of round spermatids for ROSI. In conclusion, one of the possible limitations for the low efficiency of ROSI is the ambiguous definition of a round spermatid that makes its selection difficult. Additional studies are therefore needed to standardize the selection criteria.

3.2.3. *Stage-specific molecular markers of round spermatids*

Extensive work has been performed on the identification of stage-specific markers of round spermatids in different species [262–265] for studying the molecular mechanisms regulating spermatogenesis and spermatozoa differentiation, which involve substantial morphological and cellular transformations. However, the mechanisms driving this process has not been well established yet. Moreover, although it has been shown that gene expression decreases during spermiogenesis, the current findings are still misleading. Some researchers reported that transcription does not occur in round spermatids [266] and that internal transcripts are merely a residue from spermatogenesis [267]. On the contrary, it has been recently showed that even though the transcription of several genes was downregulated, other genes were upregulated during the passage from round spermatids to elongating and from elongated spermatids to epididymal sperm in both mouse and cattle [268]. Furthermore, many mRNAs encoding proteins needed for the construction of the specialized organelles of spermatozoa are stored in the round spermatid cytoplasm and seem to be translationally repressed. These finding are also supported by the presence of free messenger ribonucleoproteins in round spermatids. On the contrary, the stored mRNAs are actively translated in elongating and elongated spermatids [269]. However, the factors that repress translation in round spermatids, have been not defined yet. It will be now described the nuclear and cytoplasmic potential markers of round spermatids that could be applied to confirm their proper identification and selection.

*S*ince nuclear DNA undergoes a complete chromatin remodeling during spermatogenesis, most of the spermatid potential markers localized in the nucleus are protein involved in the replacement of histone with protamines (Table 2). In particular, while some histone variants are detected at all the stages of spermatogenesis, others are present only in the early meiotic spermatocytes until the stage of elongated spermatids. Moreover, few others are not detected in the spermatids [270], Among the different histone variants, H3.3 is detected in all types of germ cells. Its role is to contribute to the open chromatin structure, modulating spermatid transition protein 1 (TP1) removal and protamine 1 (PRM1) incorporation [271–276]. Histone variant TH2B completely replaces somatic H2B variant during meiosis and remains the main type of H2B detected in round and elongating spermatids [277–279], suggesting that TH2B might be important for meiotic and post-meiotic germ cells.

H1 subtype variant (H1T) is exclusively transcribed in mid- and late-pachytene spermatocytes [280–282]. Studies carried out *in vitro* showed that H1T binds to H1-depleted oligo-nucleosomes significantly less

Table 2
Summary of nuclear and cytoplasm spermatid markers. For each marker, name, localization, function and reference are reported.

Marker name	Cell localization	Function	References
Histone variant H3.3	Nucleus	Chromatin remodeling and histone replacement	[271–276]
Histone variant TH2B	Nucleus	Replace somatic H2B variant during meiosis	[242,278, 279]
Histone variant H1T	Nucleus	Chromatin remodeling and histone replacement	[283,284]
Histone variant H1T2	Nucleus	Histone replacement and chromatin condensation	[288,289]
PRM1	Nucleus	Chromatin condensation	[295]
BRTD	Nucleus	Chromatin structure organization	[299,300]
29,000 Mr protein	Cytoplasm	protein secretion in Sertoli cells	[303]
SUN4	Cytoplasm	Nuclear remodeling	[304,305]
SPAG4L-2	Cytoplasm	acrosome biogenesis	[307]
Gcse factors	Cytoplasm	acrosome development	[308]
FAM71F1 and FAM71F2O	Cytoplasm	acrosome biogenesis	[309]
TMCO2	Cytoplasm	acrosome biogenesis	[310]
PRAMEY	Cytoplasm	acrosome biogenesis	[311]
TEX101	Cytoplasm	acromosome function	[312, 314–316]
SPERT	Cytoplasm	Cytosplam elimination during spermiogenesis	[318]

tightly than other somatic H1 subtypes. This is correlated with a relatively open and decondensed chromatin conformation, necessary for meiotic recombination and histone replacement [283,284]. Interestingly, H1t protein is found from pachytene spermatocytes to elongating spermatids [283–286], in which it constitutes up to 55 % of the total H1 linker histone found in the chromatin [286].

Another testis specific H1 variant is H1T2, which is detected only in the nucleus of round and elongated spermatids [287]. H1T2 is specifically localized in the apical area of polarised spermatid nuclei, suggesting a critical role in the replacement of histones by protamines and the processes of chromatin condensation [288,289].

Specific linker histone H1-like protein (HILS1) is another histone variant, whose expression changes along spermatid differentiation. Differently to what observed for H1T2, this histone was detected in the nucleus of elongating and elongated but not in that of round spermatids [290].

Protamine 1 (PRM1) [291], and protamine 2 (PRM2) [292–294] are known to be replaced to histones to increase chromatin compaction in mature sperm. are the most popular markers of spermatid stages. Their transcription starts at the spermatid stage for both *prm1* and *prm2* transcripts which are then stored until the late stage of elongated spermatids in ribosomal protein granules of cytoplasm, followed by translation into proteins [295]. This stage-specific pattern makes these proteins potential markers of round, elongating and elongated spermatids. It is also interesting to note that, in several species, including humans and cattle, scientists observed that aberrant expression of PRM1 or ratio of PRM1/PRM2 created disrupted sperm shape or impaired sperm function, negatively impacting on male fertility [296].

Recently, Liu et al. showed that Bromodomain and extra terminal motif family protein (BRTD) was prevalently expressed in round spermatids [297]. BRDT regulates the mechanisms of 3′-UTR truncation of transcripts expressed in the post-meiotic spermatid transcriptome [298]. Moreover, BRTD seems to be also involved in the chromatin structure organization as it can bind acetylated histones [299,300]. This hypothesis is supported by recent findings on Smarce 1, which is a member of the SWI/SNF family [301]. Smarce 1 has been identified as novel BRDT interacting partner in spermatids due to the colocalization of BRTD with acetylated H4 in elongating spermatids [301]. Therefore, BRTD is a crucial protein necessary for the normal progression of spermatogenesis. Manterola and colleagues demonstrated that loss of BRDT function disrupts the epigenetic state of the meiotic sex chromosome inactivation in spermatocytes, affecting the synapsis and silencing of the X and Y chromosomes [302].

The molecular markers of round spermatids represented by transcripts and proteins localized in the cytoplasm are mainly involved in controlling Sertoli cell protein secretion, acrosome biogenesis and function (Table 2).

Onoda and colleagues observed for the first time, that the 29,000 Mr protein was specifically localised in the cytosolic fraction of rat round spermatids, while was absent from the nuclear, mitochondrial, lysosomal and microsomal fractions [303]. In the same work, it was also established that the 29,000 Mr protein was involved in stimulating the secretion of various proteins, including transferrin, in Sertoli cells.

The SUN proteins are integral nuclear membrane protein characterized by a transmembrane domain constituting a bridge in between nucleus and cytoplasm [304,305]. Among SUN proteins, SUN4 was recently determined to be important for directing the shaping of the spermatid nucleus. The C-terminal SUN domain of SUN4 localizes to the perinuclear space, whereas the N-terminus is directed towards the nucleoplasm, interacting with the spermiogenesis-specific lamin B3 [306] and forms heteromeric assemblies with SUN3 regulating its expression [306]. Another SUN protein that characterizes the mechanisms underlying spermatogenesis is SPAG4L-2. This protein, discovered in 2011, was specifically detected in the testes of adult mice where its expression increases as spermatogenesis progresses [307]. This protein seems to be involved in the acrosome biogenesis. In the round spermatid it is localized in the nuclear apical region where acrosomic vesicle starts to form [307].

Another class of protein involved in the acrosome development is the germ cell specific gene (GCSE) factors [308]. Two main transcripts of these proteins have been identified in the testis: Gcse-L (1589 bp) and Gcse-S (906 bp). Gcse-S was expressed from the late stage of pachytene spermatocytes until the stage of round spermatids. Gcse-L expression was detected only in round spermatids. GCSE protein expression is different from that of its transcripts. The subcellular localization of GCSE-L proteins is dependent on the cell stage: it is in the nucleus of late pachytene spermatocytes, while, during meiosis, it is transported to the spermatid acrosome region. Whereas GCSE-S proteins are only expressed in the spermatid nucleus.

FAM71F1 and FAM71F2O are other factors involved in the acrosome biogenesis. They are testis-enriched proteins that include a RAB2B-binding domain, a small GTPase, implicated in membrane bound transport vesicles. In particular, in a recent work in mutant mice, it was observed that FAM71F1 interacts with RAB2A and RAB2B, two membrane transport-related proteins, and regulates the formation of acrosome in spermatids [309]. In Fam71f1-mutant mice, the acrosome was abnormally expanded at the round spermatid stage, likely because of enhanced vesicle transport [309].

The TMCO family consists of seven membrane proteins, named TMCO1 toTMCO7. Among these, recently, in the rat, TMCO2 was associated with the developing acrosome of spermatids in the vicinity of round spermatid nuclei and as curved lines associated with nuclei of elongated spermatids and caput epididymal spermatozoa [310]. These findings suggest that TMCO2 might be involved in the process of acrosome biogenesis, especially binding the acrosome to the nucleus, during spermiogenesis.

In cattle, few factors involved in spermatogenesis have been characterized in the spermatid cytoplasm. Among these, preferentially expressed antigen in melanoma Y-linked (PRAMEY) isoform (30 kDa) was highly expressed only in testes after puberty and in epididymal spermatozoa in bull [311]. In the same work, it was established that PRAMEY was predominantly located in the acrosome granule of spermatids, and in acrosome and flagellum of spermatozoa. Using electron microscopy, PRAMEY protein complex was specifically localized to the nucleus and to several cytoplasmic organelles, including the rough endoplasmic reticulum, some small vesicles, the inter-mitochondrial cement, the chromatid body and the centrioles, in spermatogonia, spermatocytes, spermatids and/or spermatozoa [311]. Among these spermatogenic stages, PRAMEY was highly enriched in and structurally associated with the matrix of the acrosomal granule in round spermatids and migrated with its expansion during acrosomal biogenesis. While the function of PRAMEY during spermatogenesis remains unclear, these findings suggest that PRAMEY may play an essential role in acrosome biogenesis and spermatogenesis in the bull [311]. Another potential marker of spermatids due to its role in the acrosome function is TEX101 [312,313]. This was initially identified in mice and showed a limited distribution with high expression in testis [314–316], and was found to be involved in the acrosome reaction during fertilization [317]. Moreover, because of its specific significance in the testis, TEX101 has been utilized as a biomarker for male human infertility. High expression of TEX101 was observed in spermatocytes and spermatids, but relatively lower staining was detected in spermatogonia. Moreover, this molecule was not detected in seminomas. These findings suggest that TEX101 is related to the maturation of germ cells and could be used for assessing spermatid quality once these cells are isolated from the testis before proceeding with ROSI.

Recently [318], Spermatid associated protein (SPERT), also named NURIT and CBY2, was found to be transcribed by round spermatids until their but it was absent in mature spermatozoa. Interestingly, immunogold electron microscopy revealed that the protein is restricted, since its first detectable appearance, to a unique spermatid organelle called the 'flower-like structure'. Although the precise function of this protein is

unknown, it may be involved in transporting proteins designated to be discarded via the residual bodies regulating the elimination of spermatid cytoplasm during spermiogenesis, enabling the sperm to acquire its streamlined architecture. Moreover, SPERT homologues are found in primates, pig and rodents. These findings reveal that, if the expression of SPERT will be confirmed to be exclusively detected at the spermatid stages in livestock species as well, this protein could be used as distinctive marker of these cell type.

Overall, several potential biomarkers, both at nuclear and cytoplasmic level, have been established for identification of round spermatids. Additional studies are needed to determine whether they can be effectively applied for identification of competent round spermatids and assessment of their quality before ROSI.

3.2.4. Viability of ROSI produced embryos: how far can we go?

ROSI can be successfully applied in humans and mouse [319,320]. However, in both species, pregnancy and birth rates are still low [321] [228,318,319]. Many studies evidenced that mice oocytes fertilized with ROSI need to be artificially activated because of the lack of oocyte-activating capacity in round spermatids of this species [322]. It has been also possible to obtain normal blastocysts and live offspring by spermatids injection without the bulk of the surrounding cytoplasm [323]. This indicates that spermatid genome is competent enough to support full-term development like mature spermatozoa in mouse. Ogonuki and colleagues [322] reported that when round spermatids were frozen/thawed, many of injected oocytes developed to 2 cell embryos without any artificial activation. Moreover, some of the embryos transferred into the oviduct of pseudo-pregnant females developed into full-term offspring [322]. This is maybe due to the fact that spermatids may need to somehow acquire SOAF (sperm-borne oocyte-activating factor) from spermatozoa and elongated spermatids in the same testicular suspension [322]. Finally, Lei et al. proved that normal mice pups can be obtained also after microinjection of round spermatids into oocytes stored at room temperature for 24 h [324] suggesting that these cells can be preserved before ROSI.

In domestic animals, the interest in producing embryos by ROSI substantially blow up between the end of the ‘90s and the beginning of the ‘20s. However, due to the low success rate, this technique is not considered yet among the reliable ARTs. Moreover, in these species, fully term subjects have not been delivered yet. In cattle, the first attempt was reported by Goto and colleagues [255] who obtained a relatively low blastocyst rate following injection of various type of male germ cells including spermatids into the oocytes [255]. Later, Ock and colleagues [325] improved the blastocyst formation rate reaching a level comparable to that obtained by ICSI. In the same work, the authors showed that it is possible to use frozen–thawed presumptive round spermatids to produce blastocyst *in vitro*. However, freezing steps and complexity in method separation negatively affect the success rate of ROSI. These results demonstrate that round spermatids can be easily injected into mature oocytes however these cells are fragile and the procedures used for the isolation and preparation seem to be detrimental for their quality.

In pig, the first attempts of ROSI was correlated with embryos that developed to the 2-cell stage only, although the authors demonstrated that round spermatid nuclei of the pig can develop into a morphologically normal pronucleus in matured porcine oocytes and are competent to participate in syngamy with the ootid chromatin [326]. In the same period, another group reported for the first time the development of porcine oocytes to blastocyst stage following ROSI. The authors also evidenced that it was necessary to perform an oocyte activation procedure before ROSI into the oocytes as in mice [327], humans [328] and cattle [255]. This was further investigated by Choi and colleague that proved that a 2 h oocyte activation after the round spermatid injection improved the normal fertilization and early embryo developmental rate [329]. These findings were also demonstrated in rabbit. Electrical stimulation of oocytes before spermatid injections had beneficial effects on oocytes activation. Using this procedure, the authors obtained embryos *in vitro* that developed normally through implantation and were carried successfully through complete gestation in the recipient does [328]. Recently, a systematic study was conducted on goat. In this study, the authors clearly showed that artificial activation of oocytes is essential in this species as well, since goat round spermatids can organize functional microtubular asters in activated oocytes. It is interesting to note that, in this study, the authors show that centrosome is of paternal origin because both round spermatid and sperm asters organized an extensive microtubule network after intra-oocyte injection [330]. Last but not least, nuclear DNA of round spermatids has been associated with chromatin that is less accessible and leads to impaired gene expression in the embryos [331]. Moreover, embryos have reprogramming defects at the pronuclear stages associated with the misexpression of a cohort of the genes responsible for minor zygotic genome activation [332].

Overall, these results show that ROSI cannot be applied to domestic animals, including ruminants, due to its poor success and reproducibility. This is correlated with different factors, which include the lack of a standardised protocol, the problem of oocyte activation and molecular differences of round spermatids with mature sperm. Additional studies are therefore necessary not only to improve the current protocols but also to broaden the knowledges of some aspects related to the spermatids and oocyte itself before ROSI can be successfully applied in domestics animals as a technique useful to shorten generational intervals.

4. Conclusions

Advancements in ARTs have allowed to develop several methodologies that could potentially be used to reduce the generation time in both male and female in domestic ruminants.

In females, the applications of omics technologies combined with ARTs is providing an opportunity to gain a deeper to better understand the difference between adult and prepubertal oocytes. Besides, the establishment of new IVM protocols tailored for promoting acquisition of competence, including strategies for delaying nuclear maturation and enhancing cytoplasmic maturation, the generation of 3D IVM systems have showed the potential for improving oocyte quality.

In males, several 2D and 3D culture system approaches have been developed to recreate the spermatogenesis *in vitro*. However, none have been successful so far in ruminants. Moreover, ROSI and consequent IVEP is still an unsuccessful technique in farm animals not only because of round spermatids inadequate isolation and selection procedures but also for molecular aspects related to spermatid and inefficient oocyte activation.

Overall, a substantial shortening of the generation interval is still far away in domestic ruminants. Further work is still necessary to develop suitable methodologies with the aim to generate viable embryos from neonatal animals or even before birth. If successful, these approaches would exponentially increase the power of GS to accelerate the evolution of domestic breeds towards the needs of a modern agriculture.

Data availability

Not applicable.

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CRediT authorship contribution statement

Rolando Pasquariello: Writing – original draft. **Luisa Bogliolo:** Writing – original draft. **Francesca Di Filippo:** Visualization, Methodology. **Giovanni Giuseppe Leoni:** Writing – original draft. **Stefano Nieddu:** Writing – original draft. **Andrea Podda:** Visualization, Methodology. **Tiziana A.L. Brevini:** Writing – review & editing. **Fulvio Gandolfi:** Writing – review & editing.

Declaration of competing interest

None.

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